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**Severe malaria: - Identifying immune targets and mechanisms
associated with protection in Kenyan children**

A thesis submitted for the degree of

Doctor of Philosophy

Life and Biomolecular Sciences

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Affiliated Research Centre

KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya

Collaborating Establishment

The Jenner Institute, University of Oxford

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ABSTRACT

Naturally-acquired immunity (NAI) to malaria develops first to the most severe form of disease followed by uncomplicated malaria episodes. NAI is never completely achieved therefore asymptomatic *P. falciparum* infections are often detected in adults living in malaria endemic areas. The timing and sequential acquisition of immunity to the three distinct manifestations of an infection suggests that different targets and mechanisms of protection are involved. Immunity is thought to be partly mediated by antibodies directed to blood-stage antigens. This thesis explores the potential merozoite targets and mechanisms of immunity to severe malaria and to uncomplicated malaria episodes in Kenyan children.

First, using a well-studied cohort of children aged 1-12 years recruited during a period of high malaria transmission (Chonyi), IgG responses to five merozoite antigens were quantified using a standard reference reagent. Thereafter, protective threshold concentrations were derived using a statistical approach and applied to a separate age-matched cohort (Junju) in which antibodies to the same antigens were not associated with protection using a conventional cutoff of seropositivity. Children who achieved antibody concentrations above the proposed thresholds had a reduced risk of developing clinical episodes of malaria during the subsequent 6-months of follow-up.

Second, using a matched case-control design nested within a birth cohort of children followed up every three months for the first two years of life, I examined the targets and mechanisms of protection against severe malaria. Children admitted to Kilifi County hospital with well-defined severe malaria were identified and matched to controls who were never admitted to hospital with severe malaria. Antibody responses to five merozoite antigens were measured in all the three-monthly samples from the cases and controls and prospectively associated with the odds of developing severe malaria. The

ability of antibodies to inhibit parasite growth in the Growth Inhibition Activity (GIA) assay and mediate antibody dependent respiratory burst (ADRB) by neutrophils was also investigated and prospectively associated with the odds of developing severe malaria. Dynamic antibody patterns were observed with transient peaks in antibody titres occurring during asymptomatic infections in some children. Overall, antibody levels were similar in the cases and controls and were not associated with a reduced odds of developing severe malaria with the exception of anti-AMA1 antibodies. Interestingly, children who had a combination of antibodies that mediated both GIA and ADRB, had a significantly reduced odds of developing severe malaria.

This thesis provides direct evidence of an association between achieving specific antibody thresholds and protection against uncomplicated episodes of malaria. My findings also highlight the possible involvement of multiple antibody-mediated effector functions in protection against severe malaria in young children. These findings enhance our knowledge of the acquisition of immunity to malaria and provide insights that may be valuable for advancing malaria vaccine development and testing.

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DEDICATION

Soli Deo Gloria

(S.D.G)

TABLE OF CONTENTS

ABSTRACT	2
ACKNOWLEDGEMENTS	4
DEDICATION	6
TABLE OF CONTENTS	7
LIST OF FIGURES	14
LIST OF TABLES	21
LIST OF ABBREVIATIONS	23
PUBLICATIONS	26
CHAPTER ONE	27
1.0 Introduction	27
1.1 Life cycle of <i>Plasmodium spp</i>	29
1.2 The epidemiology of <i>P. falciparum</i> malaria.....	31
1.3 Clinical features of <i>P. falciparum</i> malaria.....	34
1.4 Pathogenesis of severe <i>P. falciparum</i> malaria.....	38
1.5 Management of severe malaria.....	42
1.6 Malaria control interventions.....	43
1.6.1 Vector control strategies	43
1.6.2 Chemoprevention and chemoprophylaxis.....	45
1.7 Malaria vaccines.....	45
1.7.1 Pre-erythrocytic stage vaccines.....	46
1.7.2 Erythrocytic vaccines.....	48
1.7.3 Transmission blocking vaccines	50
1.8 Naturally-acquired immunity to malaria	52
1.8.1 The role of antibody and cellular responses in naturally-acquired immunity to malaria	55
1.8.2 Antibodies to blood-stage antigens and risk of severe malaria.....	59
1.9 <i>Plasmodium falciparum</i> merozoite antigens	73
1.9.1 Apical Membrane antigen 1 (AMA1).....	73
1.9.2 Merozoite surface protein -2 (MSP-2).....	75
1.9.3 Merozoite Surface Protein 3 (MSP-3)	77

1.9.4 Merozoite surface protein 1 (MSP-1)	78
1.9.5 <i>Plasmodium falciparum</i> reticulocyte binding-like homologue protein 2 (PfRh2).....	80
1.10 Aims and scope of the thesis	81
CHAPTER TWO	86
Materials and Methods	86
2.0 Study area	86
2.1 Study population.....	86
2.2 Study design	88
2.2.1 Kilifi Birth Cohort	88
2.2.2 Matched case-control study	89
2.2.3 Adult serum samples.....	90
2.3 Laboratory methods.....	90
2.3.1 Recombinant <i>P. falciparum</i> antigens	90
2.3.2 Enzyme Linked Immunosorbent Assay (ELISA).....	92
2.3.2.1 Standard ELISA	92
2.3.2.2 Multiplex bead-based antibody assay	93
2.3.3 <i>In vitro</i> <i>P. falciparum</i> parasite culture	95
2.3.4 Isolation of parasitophorous enclosed merozoite structures from <i>P. falciparum</i> cultures	96
2.3.5 Antibody function assays.....	97
2.3.5.1 Growth Inhibition Assays	97
2.3.5.2 Isolation of polymorphonuclear (PMN) leukocytes from whole blood ...	99
2.3.5.3 Antibody dependent respiratory burst (ADRB) assay.....	100
2.4 Cohort description	101
2.4.1 General characteristics of the KBC cohort	101
2.4.2 Summary of severe malaria cases and controls	102
CHAPTER THREE.....	108
Comparison of ELISA methods for quantifying malaria-specific antibody amounts...	108
3.0 Introduction	108
3.1 Rationale.....	110
3.2 Objective.....	111
3.3 Methods	111
3.3.1 Recombinant antigens and standard ELISA	111

3.3.2 Semi-immune adult sera	111
3.3.2.1 Pool of sera from malaria exposed Kilifi adults (PHIS)	111
3.3.2.2 Malaria Immune Globulin (MIG)	112
3.3.3 Assigning antibody units to the pooled semi-immune serum (PHIS) and MIG	112
3.3.4 Determining antibody units of test samples from a standard curve of PHIS and MIG	113
3.3.5 End point titration of individual test sera.....	113
3.3.6 Statistical analysis.....	113
3.4 Results	114
3.4.1 Assigning antibody units to PHIS and MIG	114
3.4.2 End point titration	115
3.4.3 Comparison of antibody units derived from a single dilution (1:1000) versus a full dilution curve.....	117
3.4.4 Determining an optimal single serum dilution for estimating accurate antibody concentrations from a standard curve	119
3.4.5. Determining relative antibody amounts from a standard curve of MIG.....	124
3.4.6. Comparison of antibody amounts derived from a standard curve of PHIS versus MIG	126
3.4.7 Reproducibility of the ELISA data	127
3.5 Discussion.....	128
CHAPTER FOUR.....	131
A threshold concentration of anti-merozoite antibodies is required for protection from clinical episodes of malaria	131
4.0 Introduction	131
4.1 Methods	133
4.1.1 Study population	133
4.1.1.1 Chonyi cohort.....	133
4.1.1.2 Junju cohort.....	134
4.1.2 Recombinant <i>P. falciparum</i> merozoite antigens.....	134
4.1.3 Enzyme-Linked Immunosorbent Assay (ELISA).....	135
4.1.4 Statistical analysis.....	135
4.1.4.1 Establishing the presence of a “dose-response” relationship between antibodies and outcome in the Chonyi cohort.....	136

4.1.4.2 Identifying the antibody concentrations associated with protection	136
4.1.4.3 Comparing analyses based on protective thresholds with those based on conventional cutoffs	137
4.2. Results	137
4.2.1. Effect of varying antibody concentration and protection against clinical episodes of malaria in the Chonyi cohort.	137
4.2.2. Protective efficacy of antibodies to merozoite antigens in the Chonyi cohort	138
4.2.3. Protective efficacy of antibodies to merozoite antigens in the Junju cohort	139
4.2.4 Identifying a threshold concentration of antibodies that best correlates with a reduced risk of malaria.....	143
4.2.5. Antibody concentrations are below protective thresholds in Junju cohort..	145
4.2.6. Protective thresholds explain apparent lack of protection in the Junju cohort	147
4.3 Discussion.....	148
CHAPTER FOUR (SUPPLEMENT)	152
Exploratory post-hoc analysis	152
A comparison of threshold antibody concentrations associated with protection against clinical episodes of malaria in two villages in Kilifi and in Tanzania	152
Methods	153
Results	153
Discussion.....	160
CHAPTER FIVE.....	163
Antibodies against merozoite antigens and protection against severe malaria in a longitudinally monitored birth cohort of Kenyan children	163
5.0 Introduction	163
5.1 Objectives	165
5.2 Methods	165
5.2.1 Recombinant antigens and Multiplex ELISAs.	165
5.2.2 Serum and red blood cell pellet samples.....	165
5.2.3 Detection of <i>P. falciparum</i> infections by microscopy and PCR.....	166
5.2.4 Statistical analysis.....	166
5.3 Results	167

5.3.1 Factors that influence antibody titres in children aged 0-2 years	167
5.3.2 Overall age-specific antibody prevalence.....	170
5.3.2.1 Age-specific antibody levels amongst the cases and controls.....	171
5.3.3 Individual antibody profiles in relation to the presence or absence of infection	175
5.3.4 The profiles of antibody responses for individual risk sets	178
5.3.5 Dynamics of antibody responses over the follow-up period	181
5.3.6 Association between antibodies and odds of developing severe malaria	185
5.3.7 Association between the breadth of antibody responses and odds of developing severe malaria.....	192
5.4 Discussion.....	193
CHAPTER SIX	202
The role of transplacentally acquired antibodies in protection against infection and severe malaria during the first six months of life.....	202
6.0 Introduction	202
6.1 Objectives	206
6.2 Methods	206
6.2.1 Serum samples	206
6.2.2 Recombinant antigens and Multiplex ELISAs.	206
6.2.3 Statistical analysis.....	207
6.3 Results	208
6.3.1 Descriptive analyses of mothers and infants at enrollment	208
6.3.2 Factors that influence the level of antibodies measured in cord blood.....	209
6.3.3 Proportion of children acquiring asymptomatic infections or developing severe malaria during the study period.	213
6.3.4 Decay of transplacentally acquired antibodies	214
6.3.5 Relationship between the rate of decline of cord blood titres and initial titres	217
6.3.6 Comparison of maternally transferred antibody titres in children who subsequently acquired asymptomatic parasitemia and those who remained uninfected during the first six months of life.....	221
6.3.7 Comparison of maternally transferred antibody titres in children who subsequently developed severe malaria during the first six months of life and those who did not	226

6.3.8 Maternal antibodies and subsequent risk of acquiring asymptomatic infection or developing severe malaria during the first two years of life.	231
6.3.9 Maternal antibodies and subsequent risk of developing severe malaria during the first two years of life.	236
6.3.10 Relationship between breadth of responses and protection against asymptomatic infection and severe malaria during the first six months of life	239
6.4 Discussion.....	240
CHAPTER SEVEN.....	245
The role of antibody-mediated effector functions in protection against severe malaria	245
7.0 Introduction	245
7.1 Objectives	248
7.2 Methods	249
7.2.1 Serum samples	249
7.2.2 GIA and ADRB assays	249
7.2.3 Statistical analysis	249
7.3 Results	250
7.3.1 ADRB assay development	250
7.3.1.1 A comparison of ADRB activity induced by merozoites isolated using two techniques.....	251
7.3.1.2 Quantifying the number of PEMS isolated and added to individual wells.	253
7.3.1.3 The effect of repeated freeze-thawing on ADRB activity.....	255
7.3.1.4 The effect of donor dependent variability on ADRB activity	257
7.3.1.5 Comparing ADRB activity of coated PEMS versus PEMS in solution.....	258
7.3.1.6 Determining the optimal coating concentration of PEMS preparations	260
7.3.1.7 Determining the optimal serum dilution	261
7.3.1.8 Determining ADRB inter-assay variability.....	262
7.3.2 Comparison of ADRB activity between malaria-exposed children and adults versus malaria non-exposed adults	263
7.3.3 Age-specific ADRB activity in infants aged 0-2 years.....	264
7.3.4 Correlation between ADRB activity and antibody titres against specific antigens in adults	266

7.3.5 Correlation between ADRB activity and antibody titres to specific antigens in children who developed severe malaria (cases) and those who did not (controls).	267
7.3.6 Comparing growth inhibition of <i>P. falciparum</i> 3D7 parasites by human sera in a one-growth cycle assay (pLDH detection) versus a two-growth cycle assay (flow cytometry)	270
7.3.7 Age-specific growth inhibition levels in children aged 0-2 years.	273
7.3.8. Association between GIA and antibody titres against specific antigens in adult serum samples	274
7.3.9 Association between GIA and antibody titres against specific antigens in plasma samples collected from children aged 0-2 years	276
7.3.10 Correlation between GIA and ADRB	279
7.3.11 A comparison between the breadth of antibody responses with ADRB and GIA in children aged 0-2 years	280
7.3.12 The association between ADRB and GIA and protection against severe malaria during the first two years of life	281
7.4 Discussion	282
CHAPTER EIGHT	288
Conclusions and recommendations	288
8.0 Summary of findings	288
8.1 Recommendations for future studies	291
REFERENCES	294

LIST OF FIGURES

Figure 1.1: The life cycle of *Plasmodium spp.*

Figure 1.2: Estimated *Plasmodium falciparum* parasite prevalence endemicity maps in the year 2000 (A) and 2010 (B) in Africa.

Figure 1.3: The prevalence, overlap and mortality of the major clinical symptoms of severe malaria in a rural hospital in coastal Kenya.

Figure 2.1: Map of Kilifi District showing the administrative locations covered by the KHDSS

Figure 2.2: A schematic diagram of the Kilifi Birth Cohort illustrating the selection of cases and controls for the matched case-control study.

Figure 2.3: The prevalence and overlap of the major severe malaria symptoms among the cases.

Figure 2.4: Map of KHDSS showing the number of KBC severe malaria cases recorded from each administrative location during the follow-up period.

Figure 3.1: Standard curves of a semi-immune serum pool (PHIS) against AMA1, MSP-3, MSP-2 and MSP-1₁₉

Figure 3.2: End point titration curves of 22 sera tested against (A) AMA-1 (B) MSP-3 (C) MSP-2 and (D) MSP-1₁₉

Figure 3.3: Comparison of end point titers calculated from a single dilution point (1:1000) versus a full dilution curve of individual samples.

Figure 3.4.1: AMA-1 end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis).

Figure 3.4.2: MSP-1₁₉ end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis).

Figure 3.4.3: MSP-2 end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis).

Figure 3.4.4: MSP-3 end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis).

Figure 3.5: Serial dilution curves of MIG tested against eight merozoite antigens.

Figure 3.6: Individual dilution curves (1:1000 to 1:1024000) of twenty two adult serum samples and a malaria specific standard reference reagent (MIG) represented as a black bold line.

Figure 3.7: Comparison of end point titers derived from a standard curve of PHIS and relative amounts derived from a standard curve of MIG.

Figure 3.8: Reproducibility of ELISA data.

Figure 4.1: Protective efficacy of antibodies based on different cutoff levels. Antibody titers were divided into quartiles (25th, 50th and 75th) based on the distribution of antibody amounts in the Chonyi cohort.

Figure 4.2: Distribution of antibody concentrations in age-matched children recruited from the Chonyi and Junju (grey) cohorts against (A) AMA1, (B) MSP-2 and (C) MSP-3.

Figure 4.3: Plots of goodness of fit log likelihood estimates and the dichotomization point for the range of antibody titres obtained for (A) AMA1 (B) MSP-3 (C) MSP-2 (D) GLURP-R2 (E) GLURP-R0 and (F) MSP-1₁₉ in the Chonyi cohort.

Figure 4.4: Plots of goodness of fit log likelihood estimates and the dichotomization point for the range of antibody titres obtained for (A) AMA1 (B) MSP-3 (C) MSP-2 in the moderate transmission Junju cohort.

Figure 5.1: Age- specific antibody prevalence in children aged 0-2 years of age.

Figure 5.2: The level of antibodies to merozoite antigens by age in children aged 0-2 years.

Figure 5.3: Individual profiles of IgG responses to merozoite antigens tested.

Figure 5.4: Representative patterns of IgG responses to merozoite antigens in defined risk sets.

Figure 5.5.1: Antibody profiles over the follow-up period categorized as being relatively stable or dynamic.

Figure 5.5.2: Proportion of cases and controls demonstrating dynamic versus stable responses over the follow-up period, to each antigen tested.

Figure 5.6: Illustration of the different time points that an antibody titre measurement was taken and used to calculate the protective efficacy of specific antibodies against severe malaria.

Figure 5.7: The breadth of anti-merozoite antibody responses in cases and controls in (A) and the protective efficacy of increasing breadth of antibody responses in (B).

Figure 6.1: Kaplan-Meier curves of (A) time to first infection and (B) time to severe malaria episode in children who were recruited at birth and had a cord blood sample taken.

Figure 6.2: Scatter plots of antibody titres to (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *PfRh2* during the first six months of life.

Figure 6.3: Scatter plots of antibody titres to (A) AMA1(FVO), (C) AMA1(3D7), (E) MSP-2(Dd2), (G) MSP-2(CH150/9), (I) MSP-3(3D7), (K) MSP-3(K1), (M) MSP-1₁₉ and (O) *PfRh2*, showing the decay rates according to the initial (cord blood) titres. The decay rates and 95% CI's of cord blood titres (on the y-axis) according to the different quartile levels (on the x-axis) are shown for the antigens tested (B) AMA1 (FVO), (D) AMA1 (3D7), (F) MSP-2(Dd2), (H) MSP-2(CH150/9), (J) MSP-3(3D7), (L) MSP-3(K1), (N) MSP-1₁₉ and (P) *PfRh2*.

Figure 6.4.1: Cord blood antibody levels against (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *PfRh2* in children who subsequently acquired infections (circles) and those who did not (squares), during six months of follow-up.

Figure 6.4.2: Scatter plots of the distribution of individual antibody half-lives to (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *PfRh2* in children who subsequently acquired infections and those who did not during six months of follow-up.

Figure 6.4.3: Cord blood antibody levels against (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *PfRh2* in children who subsequently developed severe malaria and those who did not during six months of follow-up.

Figure 6.4.4: Scatter plots of the distribution of individual antibody half-lives to (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-

3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *Pf*Rh2 in children who subsequently developed severe malaria and those who did not during six months of follow-up.

Figure 6.5: The breadth of anti-merozoite antibody responses in cord blood plasma samples from children who acquired asymptomatic infections during the first six months of life versus those who did (A) and from children who developed severe malaria during the first six months of life versus those who remained disease free (B).

Figure 7.1.1: ADRB activity induced by three different merozoite preparations against a panel of sera.

Figure 7.1.2: ADRB activity induced by PEMS frozen at a concentration of 1.85×10^5 schizonts/ml and pure isolated merozoites in two independent experiments.

Figure 7.1.3: The effect of repeated freeze-thawing of PEMS on ADRB activity.

Figure 7.1.4: ADRB induction by PMNs obtained from different donors.

Figure 7.1.5: A comparison between ADRB activity induced by coated PEMS versus PEMS in solution.

Figure 7.1.6: Determining the optimal coating concentration of PEMS.

Figure 7.1.7: Determining the optimal serum dilution.

Figure 7.1.8: ADRB assay reproducibility.

Figure 7.2: Comparison of ADRB levels induced by serum obtained from adults resident in Kilifi County, children aged 0-2 years and malaria naïve adults.

Figure 7.3: Age-specific ADRB levels in infants aged 0-2 years.

Figure 7.4: Correlation analysis between ADRB levels and antibody titres against (A) AMA1 (HB3), (B) MSP-2 (Dd2), (C) MSP-3 (3D7) and (D) GLURP-R0 fragment measured in plasma samples collected from adults resident in Kilifi (N=75).

Figure 7.5: Correlation analysis between antibody titres to specific antigens and ADRB activity in children who developed severe malaria (cases) versus their corresponding controls.

Figure 7.6.1: A comparison between GIA levels assessed by measuring pLDH production following a single growth cycle or flow cytometry analysis of ethidium bromide stained parasites following two growth cycles.

Figure 7.6.2: Correlation analysis between growth inhibition determined by measuring pLDH production after one growth cycle (y-axis) or by flow cytometry after two growth cycles (x-axis) in plasma samples collected from children aged 0-2 years (A) and adults resident in Kilifi county (B).

Figure 7.6.3: Distribution of GIA levels of plasma obtained from adults resident in Kilifi County, children aged 0-2 years recruited into a birth cohort and malaria naïve adults resident in the UK.

Figure 7.7: Age-specific GIA levels in infants aged 0-2 years.

Figure 7.8: Correlation between GIA levels quantified by flow cytometry in a two growth cycle assay and antibody titres against (A) AMA1 (HB3), (B) MSP-2 (Dd2), (C) MSP-3 (3D7) and (D) GLURP-R0 fragment measured in plasma samples collected from adults resident in Kilifi (N=75).

Figure 7.9: Correlations between antibody titres to specific antigens and GIA activity measured in a one growth cycle assay in children who developed severe malaria (cases) versus their corresponding controls.

Figure 7.10: The correlation between ADRB activity and the 2-cycle growth inhibition assays in children who subsequently developed severe malaria (A) versus their matched controls (B) and adults resident in Kilifi County (C).

Figure 7.11: Correlation between breadth of antibody responses and GIA levels (A) or ADRB levels (B) in children aged 0-2 years.

LIST OF TABLES

Table 1: A summary of hospital-based case-control studies examining the association between antibodies to *P. falciparum* blood stage antigens and protection against severe malaria.

Table 2.1: Details of the recombinant *P. falciparum* antigens used in the study.

Table 2.2: Baseline characteristics of selected cases and controls illustrating the success of matching.

Table 4.1: A comparison of analyses based on antibody seropositivity, high antibody levels and protective thresholds in the Junju Cohort.

Table 4.2: Cutoff values for antibody seropositivity, high antibody levels and protective thresholds in the Chonyi Cohort.

Table 4.3: Protective threshold concentrations and the 95% confidence interval estimates derived independently in the Chonyi, Junju and Tanzanian cohorts.

Table 4.4: A comparison of the protective efficacy of cutoffs defined and applied independently in three different settings (Chonyi, Junju and Tanzania).

Table 5.1: The influence of different factors on the IgG response to a panel of merozoite antigens measured at three-monthly sampling points from birth upto two years of age.

Table 5.2: The proportion of risk sets classified into three groups according to the profile of antibody responses for each antigen tested.

Table 5.3.1: The protective efficacy of antibodies measured at one time point prior to admission, against severe malaria.

Table 5.3.2: Protective efficacy of the mean antibody titres of measurements obtained at multiple time point prior to admission, against severe malaria.

Table 6.1: The role of transplacentally acquired antibodies in protection against malaria in infants.

Table 6.2: Baseline characteristics of mothers and infants at enrolment.

Table 6.3: Factors that influence the levels of antibodies measured in cord blood samples against a panel of merozoite antigens.

Table 6.4: The rate of decay and antibody half-lives of maternally transferred antibodies to specific antigens tested.

Table 6.5.1: The protective effect of specific-antibodies against acquisition of asymptomatic parasitemia during the first six months of life. Analyses are based on antibody seropositivity and protective thresholds.

Table 6.5.2. The protective effect of specific-antibodies against acquisition of asymptomatic parasitemia during the first two years of life. Analyses are based on antibody seropositivity and protective threshold cutoffs.

Table 6.6.1: The protective effect of specific-antibodies against development of severe malaria during the first six months of life. Analyses are based on antibody seropositivity and protective thresholds.

Table 6.6.2: The protective effect of specific-antibodies against development of severe malaria during the first two years of life. Analyses are based on antibody seropositivity and protective threshold cutoffs.

LIST OF ABBREVIATIONS

ACT	Artemisinin based Combination Therapy
ADCI	Antibody Dependent Cellular Inhibition
ADRB	Antibody Dependent Respiratory Burst
AMA	Apical Membrane Antigen
APAD	3-Acetylpyridine Adenine Dinucleotide
AU	Arbitrary Units
BCS	Blantyre Coma Score
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CHMI	Controlled Human Malaria Infection
CM	Cerebral Malaria
CSA	Chondroitin Sulphate A
CSP	Circumsporozoite Protein
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DPT	Diphtheria Pertussis Tetanus
EBA	Erythrocyte Binding Antigen
EDTA	Ethylenediaminetetraacetic acid
EDAC	1-ethyl-3-[3-dimethylaminopropyl) carbodiimide hydrochloride
EIR	Entomological Inoculation Rate
ELISA	Enzyme Linked Immunosorbent Assay
EPI	Expanded Programme on Immunization
GIA	Growth Inhibition Assay

GLURP	Glutamate Rich Protein.
GMP	Good Manufacturing Practice
GPI	Glycosylphosphatidylinositol
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
ICAM1	Intercellular Adhesion Molecule 1
IE	Infected Erythrocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
IQR	Interquartile range
IV	Intravenous
LSA	Liver Stage Antigen
MBP	Maltose Binding Protein
MFI	Median Fluorescence Intensity
MSP	Merozoite Surface Protein
MVA	Modified Vaccinia Ankara
MWCO	Molecular Weight cut-off
NBT	Nitro Blue Tetrazolium
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
<i>Pf</i> EMP1	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein 1
pLDH	Parasite lactate dehydrogenase
PMN	Polymorphonuclear
RESA	Ring-infected Erythrocyte Surface Antigen
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SERA	Serine Rich Antigen
SM	Severe Malaria
SMA	Severe Malarial Anemia
SMC	Seasonal Malaria Chemoprevention
S-NHS	sulfo-N-hydroxysulfosuccinimide
SP	Sulphadoxine Pyrimethamine
SSP	Sporozoite Surface Protein
STARP	Sporozoite Threonine and Asparagine-Rich Protein
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
TNF	Tumor Necrosis Factor
TRAP	Thrombospondin Related Adhesive Protein
VEGF	Vascular Endothelial Growth Factor
VSA	Variant Surface Antigen
WHO	World Health Organisation

PUBLICATIONS

Murungi L.M., Kamuyu G., Lowe B., Bejon P., Theisen M., Kinyanjui S.M., Marsh K., Osier F.H. (2013) A threshold concentration of anti-merozoite antibodies is required for protection from clinical episodes of malaria. Vaccine Aug 20; **31**(37):3936-42

Lundblom K., **Murungi L.**, Nyaga V., Olsson D., Rono J., Osier F., Ogada E., Montgomery S., Scott J.A., Marsh K., Farnert A. (2013) *Plasmodium falciparum* infection patterns since birth and risk of severe malaria: a nested case-control study in children on the coast of Kenya. PLoS One **8**(2): e56032

CHAPTER ONE

1.0 Introduction

Malaria is a global public health scourge affecting approximately 3.4 billion people worldwide. The World Health Organization estimates that in the year 2012, approximately 207 million cases were reported accounting for 627,000 deaths (WHO 2013). A disproportionate number of the malaria-related deaths occur in sub-Saharan Africa with pregnant women and children under the age of 5 being most severely affected. Despite the grave outlook, a tremendous achievement towards reducing the malaria burden has been reported in several countries over the past decade, with mortality rates declining by 42% globally in all age groups and by 54% in African children aged five and below, since the year 2000 (WHO 2013). Huge financial investments in expansion and coverage of malaria control programmes particularly in resource-poor settings have contributed to this reduction, although changing climate patterns, improved living conditions, increased urbanization and human settlement, have also played a role (Noor et al. 2014). Large-scale deployment of insecticide treated bed nets (ITNs) has risen significantly from 3% to 54% between the year 2000 and 2013 contributing to an increase in the proportion of protected populations. Among individuals seeking care at health facilities, accurate diagnosis of suspected malaria cases has risen from 44% to 64% and this is attributable to the availability of rapid diagnostic tests (RDTs) for screening. In the year 2007, the WHO recommended the uptake of highly effective artemisinin based combination (ACT) drugs in health facilities as first-line treatment for malaria and this has been adopted by at least seventy nine countries (WHO 2013).

Although the benefits of the existing malaria control measures are unprecedented, only 52 out of 103 countries are on track towards realizing the goal of the Global Malaria

Action Plan initiative, which is to reduce malaria incidence rates by three quarters, by the year 2015 (WHO 2013). Furthermore, these countries accounted for only 8 million (3.5%) of the 226 million reported malaria cases in the year 2000, which is an indication that the war against malaria is far from being achieved. In addition, several countries have also reported the emergence of parasites resistant to ACT drugs (Wongsrichanalai and Meshnick 2008, Noedl et al. 2008, Dondorp et al. 2009) and increased resistance of malaria vectors to commonly used pyrethroids (Ranson et al. 2011, Butler 2011), which threatens the ability to sustain malaria control interventions. A multifaceted approach involving the sustenance and expansion of available malaria control approaches, the search for more effective drugs, monitoring of emerging drug resistance to the available ACTs, better vector management tools and the search for more effective preventative options such as vaccines, is key to achieving the current drive towards malaria elimination and eradication.

To date, vaccination remains the most cost effective method of reducing the burden of diseases particularly in areas with limited resources (Ehreth 2003). Widespread vaccination programmes have led to the eradication of small pox and are currently successful against the prevention of polio, measles, hepatitis and *Haemophilus influenzae*, amongst others. Despite concerted efforts over the years to develop vaccines against parasitic infections, there is as yet no licensed malaria vaccine. This is not surprising given the complex life cycle of the malaria parasite both in human and mosquito hosts and the numerous proteins expressed during the different developmental stages. The most advanced malaria vaccine, currently undergoing Phase III trials in paediatric populations in Africa has only achieved modest efficacy of 30% and 50% against severe and clinical episodes of malaria, respectively (Agnandji et al. 2011, Agnandji et al. 2012) with waning protective efficacy over a four-year follow-up period (Olotu et al. 2013).

1.1 Life cycle of *Plasmodium* spp

Malaria is caused by infection with the obligate intracellular parasites of the *Plasmodium* species transmitted via the bite of an infected female anopheline mosquito. The four species of *Plasmodium* that infect humans are *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae*. Recently, the monkey parasite *P. knowlesi* (that is morphologically indistinguishable from *P. malariae*) was identified in individuals living in close proximity to macaque monkeys (Singh et al. 2004) and has since been established as the fifth human *Plasmodium* species (White 2008).

The life cycle of *Plasmodium* spp involves an asexual cycle in the human host and a sexual cycle in the mosquito vector (Figure 1.1). Infection in the human host begins with the bite of an infected female anopheline mosquito, which injects sporozoites under the skin. The motile sporozoites penetrate the blood vessels and are carried to the liver where they cross the liver endothelium and invade hepatocytes (Sinnis and Coppi 2007). Quantitative imaging studies using fluorescently labelled *P. berghei* parasites in mice have shown that a proportion of sporozoites also migrate from the dermis to the proximal draining lymph node and are subsequently destroyed by resident dendritic cells (Amino et al. 2006). Inside the liver, a clinically silent stage, sporozoites multiply and differentiate into thousands of merozoites enclosed within liver schizonts. Following schizont rupture, the merozoites are confined within vesicles known as merosomes, which bud off from the hepatocytes into the liver sinusoids releasing the merozoites into circulation (Sturm et al. 2006). *P. vivax* and *P. ovale* infections are characterized by dormant hypnozoites which persist in the liver causing relapses that occur up to a year after the primary infection (White 2011).

Once the merozoites are released into circulation, they attach to and invade red blood

cells to initiate the intra erythrocytic phase of development that lasts upto 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, 72 hours for *P. malariae* and a shorter period of 24 hours for *P. knowlesi*. Upon erythrocyte invasion, the merozoite differentiates into the ring-stage, then the trophozoite stage and finally undergoes asexual replication to form mature schizonts containing merozoites. The schizonts rupture and release free merozoites which re-invade erythrocytes to initiate a repeat cycle of erythrocytic development. A small proportion of blood-stage parasites differentiate into sexual forms (male and female gametocytes) that can be taken up by female anopheles mosquitoes during feeding. In the mosquito gut, the male and female gametocytes are released from infected red blood cells and develop into male and female gametes. The male and female gametes fuse to form diploid zygotes, which develop into motile ookinetes that traverse the mosquito midgut wall and form oocysts. The oocysts undergo division to produce thousands of haploid sporozoites, which are released upon oocyst rupture and migrate to the salivary glands. The cycle of human infection is re-initiated when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the dermis of the skin.

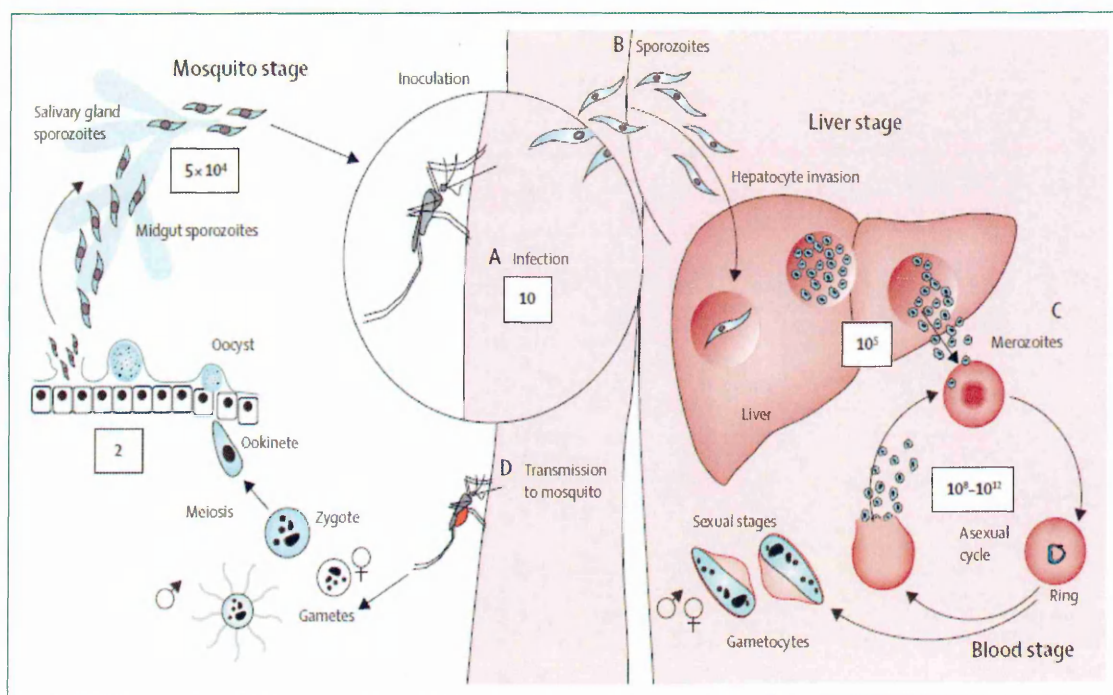


Figure 1.1 The life cycle of *Plasmodium* spp. The diagram shows the developmental stages of the parasite in the human and mosquito hosts. The estimated numbers of parasites at each stage of the life cycle are shown in boxes. Figure adopted from (White et al. 2014b).

1.2 The epidemiology of *P. falciparum* malaria

During the past decade, the epidemiology of malaria has been changing with substantial reductions in malaria transmission (Noor et al. 2014) and the number of clinical cases reported in some countries (Okiro et al. 2007, O'Meara et al. 2008, Ceesay et al. 2010, Brasseur et al. 2011, Bouyou-Akotet et al. 2009, Mmbando et al. 2010, Otten et al. 2009), although this phenomenon is not homogenous in all countries (Okiro et al. 2009, Okiro et al. 2011, Okiro et al. 2013, Roca-Feltrer et al. 2012). For instance, in Malawi,

sampling of four hospitals revealed that the number of admissions with malaria increased or remained unchanged over a ten-year period, despite uptake of malaria intervention measures (Okiro et al. 2013). A similar scenario of increase in malaria admissions from 1999 to 2009 of between 47% and 350% in five hospitals in Uganda has also been reported (Okiro et al. 2011).

Recent parasite prevalence estimates compiled from African countries show that at least 26.7% of people have transitioned into a lower endemicity class in the year 2010 compared to 2000 (Figure 1.2). The proportion of individuals living in areas of high transmission (classified as hyper or holoendemic) has also declined by 16.1% and most strikingly, the majority (87%) of the total population living in the two highest endemicity classes are concentrated in only ten countries (Noor et al. 2014). It is clear, therefore, that despite the improvements generally noted in the continent in terms of declining malaria transmission, these findings cannot be extrapolated to all endemic settings.

Spatial variations in malaria transmission at the microepidemiological level have been reported in several countries classified as having low (Noor et al. 2011) or moderate to high transmission (Bousema et al. 2010, Bejon et al. 2010, Kobayashi et al. 2012, Gaudart et al. 2006). These studies have found that particular regions/villages have relatively more febrile malaria cases, higher parasite prevalence or higher antibody seropositivity, classifying them as “hotspots” of malaria transmission. Some of the hotspots are stable over time (Bejon et al. 2010, Ernst et al. 2006) whereas others have only been identified during a single survey (Bousema et al. 2010, Bejon et al. 2011b). Targeted intervention strategies in “hotspot” areas should have a greater impact on malaria transmission.

Reflecting the decline in transmission that has occurred, there has been a significant

decrease in the overall number of clinical cases reported in several national health facilities. For instance, in Senegal, there was a 30-fold decrease in number of episodes over a 15-year period (Brasseur et al. 2011). Similarly, in Kenya and The Gambia, the number of paediatric admissions with malaria fell from 18.43 per 1000 children in 2003 to 3.42 in 2007 (O'Meara et al. 2008) and by 74%, 69% and 27% at three sites (Ceesay et al. 2010), respectively. However, the incidence of malaria in some countries still remains high and appears to be increasing (Okiro et al. 2011, Okiro et al. 2009, Okiro et al. 2013). The changes in morbidity have been paralleled by changes in the age-pattern of malaria admissions with a shift in cases to older ages (O'Meara et al. 2008). The pattern of clinical disease varies by age and transmission intensity, whereby in areas of high endemicity, the burden of disease is greatest in children under the age of 5 years whereas in areas of low transmission intensity, both children and adults bear the brunt of disease (Carneiro et al. 2010). Using mathematical models of data collected from 9 countries, Griffin *et al.* predicted that 57% of malaria cases were observed in children under 5 years of age in areas of high transmission (60% parasite prevalence in 2-10 year olds) whereas the estimates were reduced to 10% in areas of low transmission (5% parasite prevalence in 2-10 year olds) (Griffin, Ferguson and Ghani 2014). A similar pattern has also been reported in a systematic review and pooled analysis of 29 studies showing that this trend is independent of the seasonality patterns observed in the different settings (Carneiro et al. 2010). The general consensus therefore is that the brunt of disease continues to be borne by the younger age groups and intervention strategies need to be tailored depending on the epidemiological context taking into account the observed age-patterns of mortality and morbidity as malaria transmission continues to decline.

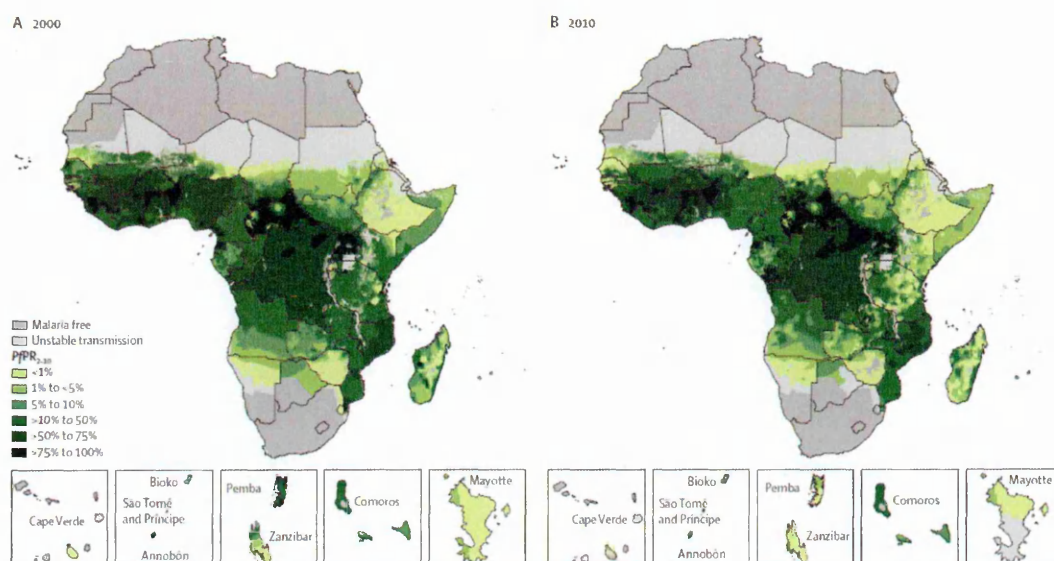


Figure 1.2. Estimated *Plasmodium falciparum* parasite prevalence endemicity maps in the year 2000 (A) and 2010 (B) in Africa. The parasite prevalence rate in children aged 2 – 10 years ($PfPR_{2-10}$) was used to define the different endemicity classes as follows: $>75\%$ $PfPR_{2-10}$ (holoendemic), $>50\% - 75\%$ $PfPR_{2-10}$ (hyperendemic), $>10\% - 50\%$ $PfPR_{2-10}$ (mesoendemic), $1\% - 10\%$ $PfPR_{2-10}$ (hypoendemic) and $<1\%$ $PfPR_{2-10}$ (low stable endemic). Figure adopted from (Noor et al. 2014).

1.3 Clinical features of *P. falciparum* malaria

After the onset of an infection, several host, parasite and environmental related factors influence disease progression and outcome (Miller et al. 2002). The initial symptoms of malaria are variable, non-specific and clinically indistinguishable from those of flu and other common causes of fever. Patients often present with intermittent fever, headache, malaise, muscle aches and occasionally abdominal pains and diarrhea. In very young children, there's increased irritability, vomiting and refusal to eat (Crawley et al. 2010). In the absence of proper diagnosis and treatment, *P. falciparum* infections can progress

to severe life-threatening malaria characterized by impaired consciousness (prostration or coma), seizures, respiratory distress, severe anemia, hypoglycaemia, metabolic acidosis and hyperlactataemia (Marsh et al. 1995, WHO 2012). These symptoms are frequently seen in both children and adults but pulmonary oedema, renal failure and severe jaundice are mainly restricted to adults. The spectrum of clinical symptoms of severe malaria varies depending on host age and malaria transmission intensity. In areas of high malaria transmission intensity, severe disease occurs in children less than 2 years often presenting with severe malaria anemia (Hb <5g/dL) whereas in areas of moderate to low transmission, severe malaria peaks at a later age (3-5 years) often presenting as cerebral malaria (CM) (impaired consciousness, coma or prostration) in this age group (Roca-Feltrer et al. 2010, Reyburn et al. 2005, Snow et al. 1994). According to the WHO guidelines, cerebral malaria is defined as unarousable coma after 1 hour of termination of a seizure in a patient with *P. falciparum* parasitemia, excluding other causes of encephalopathy (WHO 2012). The depth of coma in patients is assessed based on the verbal, motor and gaze responses on a scale of 0 to 5, referred to as the Blantyre coma score (BCS). Unarousable coma is defined as a BCS<3 and impaired consciousness as a BCS<5. Histological sections obtained from postmortem samples of fatal CM cases demonstrated the sequestration of infected red blood cells within the brain blood vessels in a majority and thus associated with hypoxia, hypovolemia and brain hemorrhages (Taylor et al. 2004). However, amongst the clinically diagnosed CM cases, 23% of those who died showed no evidence of parasite sequestration underscoring the lack of specificity of clinical diagnosis of cerebral malaria. Recent descriptions of retinal changes can reliably confirm CM cases and have successfully correlated severity of the retinal signs with fatal disease outcome (Beare et al. 2004, Lewallen et al. 1996, Hero et al. 1997). Prolonged multiple seizures are a prominent feature of cerebral malaria that often result in death occurring within 24

hours of admission in 20% of cases or residual neurological sequelae that includes ataxia, speech and hearing disorders, epilepsy, cortical blindness or hemiplegia occurring in 10% of the cases (Carter et al. 2005, Idro, Jenkins and Newton 2005, Idro et al. 2007). Severe malarial anemia (SMA) defined as a hemoglobin concentration of $<5\text{g/dL}$ of blood in the presence of detectable *P. falciparum* parasites is the most frequent presenting feature in very young children (aged 1 year) in areas of high transmission and in children aged 2 years in areas of low and stable transmission (Reyburn et al. 2005). The pathophysiology of SMA could directly or indirectly involve destruction of infected and non-infected red blood cells, suppression of erythropoiesis or dysregulation of inflammatory mediators (Reviewed in (Perkins et al. 2011). Respiratory distress, a manifestation of severe malaria which is characterized by deep breathing with in-drawing of the bony structures of the lower chest walls is a clinical indicator of metabolic acidosis (Marsh et al. 1995). Owing to increased metabolic demands, children with metabolic acidosis also develop hypoglycaemia (blood glucose $<2.2\text{mmol/l}$). Impaired vaso perfusion that is thought to occur due to sequestration of parasites in the microcirculation leads to accumulation of lactic acid, released during anaerobic glycolysis. The prognosis is often fatal particularly in children who also present with impaired consciousness (Dondorp et al. 2008, Marsh et al. 1995). The clinical features of pulmonary oedema in adults are similar to acute respiratory distress syndrome. Patients present with increased respiratory rates, increased pulmonary capillary permeability and reduced arterial oxygen levels, which results in hypoxia related signs such as convulsions and impaired consciousness. Pulmonary oedema has a case fatality rate of greater than 80%. Acute renal failure, a fairly common complication seen in adults and older children presenting to hospital with severe malaria (Dondorp et al. 2008) occurs due to tubular necrosis, a reversible condition in those who survive. Since severe malaria is a multi-organ, multi-system disorder, patients frequently present

with overlapping symptoms, of which, respiratory distress is the leading prognostic marker for fatal outcome (Marsh et al. 1995, Dondorp et al. 2008, Kendjo et al. 2013).

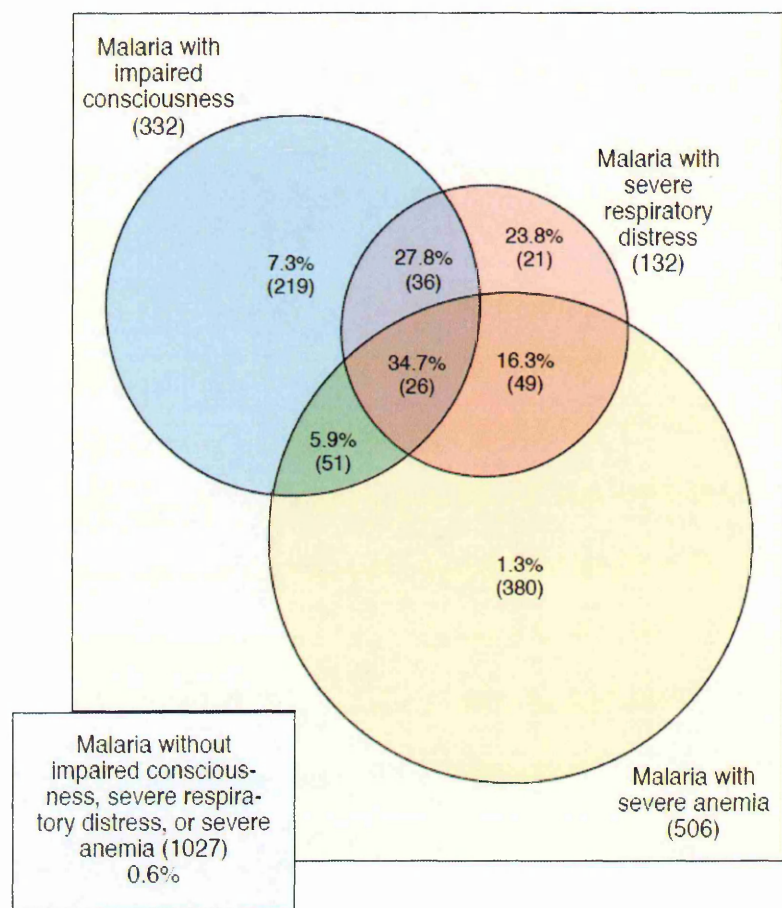


Figure 1.3 The prevalence, overlap and mortality of the major clinical symptoms of severe malaria in a rural hospital in coastal Kenya. Figure adopted from (Marsh et al. 1995).

Malaria in pregnancy is also common in malaria endemic areas particularly during first pregnancies, predisposing the infant to congenital malaria, low birth weight, premature delivery and stillbirth (Desai et al. 2007, Shulman and Dorman 2003). The risk of infant death is greater if maternal malaria occurs during the final trimester of pregnancy. Maternal anemia is the most frequent symptom seen during pregnancy although hypoglycemia and pulmonary oedema are also observed in a proportion of cases.

1.4 Pathogenesis of severe *P. falciparum* malaria

Host age, level of immunity, parasite virulence, parasite multiplication rate, antigenic variation, human genetic and environmental factors are important determinants of disease outcome and progression following an infection (Miller et al. 2002). The pathophysiology of severe malaria is complex and often comprises multiple and/or ill-defined mechanisms although the most consistent observations involve sequestration of mature parasites in the microvasculature of the most vital organs (Taylor et al. 2004, MacPherson et al. 1985, Silamut et al. 1999) and inflammation related pathology (reviewed in (Schofield and Grau 2005, Mackintosh, Beeson and Marsh 2004, Miller et al. 2002). Opinions are divided over which of these two represent the major driving force leading to disease and death.

Parasite proteins expressed on the surface of mature infected red blood cells, of which *P. falciparum* erythrocyte membrane protein -1 (PfEMP1) is the most studied, mediate binding to receptors expressed on the endothelial lining of various organs such as the brain, kidney, lung, liver and also modify the RBC surface reducing its flexibility and ability to circulate (Dondorp, Pongponratn and White 2004). In the brain and placenta, upregulation of expression of intercellular adhesion molecule 1 (ICAM1) (Turner et al.

1994) and chondroitin sulfate A (CSA) (Fried and Duffy 1996, Salanti et al. 2003) respectively, promotes cytoadherence of infected red blood cells. Parasites that sequester in the placenta express a unique variant of *Pf*EMP1 known as var2csa. Sequestration also leads to widespread endothelial activation via mechanisms that are not fully understood but several authors have identified angiogenic factors such as angiopoietin-2, Von Willebrand factor (both released from secretory vesicles known as weibel-palade bodies), soluble ICAM1 and vascular endothelial growth factor (VEGF) as important markers of disease severity (Kim et al. 2001, Oh et al. 1999, Yeo et al. 2008, Tchinda et al. 2007). In *in vitro* experiments, addition of VEGF increased expression of ICAM-1 and Ang-2 in endothelial cells (Oh et al. 1999, Kim et al. 2001). Increased levels of angiopoietin-2 were also associated with fatal outcome, increased parasite biomass, increased levels of ICAM1 and increased endothelial activation in Thai adults (Yeo et al. 2008, Lovegrove et al. 2009), Ugandan children (Lovegrove et al. 2009) and Malawian children (Conroy et al. 2012) with cerebral malaria. Plasma levels of Von Willebrand factor, a marker of endothelial activation were also elevated in children with severe malaria and were positively correlated with lactate levels, a biochemical marker of disease severity (Hollestelle et al. 2006). Endothelial activation can be reversed by L-arginine and nitric oxide in *in vitro* experiments (De Caterina et al. 1995) whereas *in vivo*, low levels of both are associated with disease severity in children (Anstey et al. 1996, Lopansri et al. 2003) and adults (Yeo et al. 2007). These findings suggest that nitric oxide and L-arginine could be potential therapeutic agents for treatment of cerebral malaria but clinical trials of both as possible adjunct therapies have not been successful (reviewed in (John et al. 2010).

Parasite toxins and red blood cell (RBC) debris released during schizont rupture such as glycosylphosphatidylinositol (GPI) and hemozoin (product of heme breakdown) interact with toll like receptors on macrophages and dendritic cells mediating excessive release

of inflammatory mediators TNF- α , IL-12, IL-18 and nitric oxide (Coban et al. 2005, Gowda 2007, Nebl, De Veer and Schofield 2005). The accumulation of monocytes, macrophages (Patnaik et al. 1994), platelet and fibrinogen deposition (Grau et al. 2003) and microparticles (vesicles released from the cell membrane of apoptotic or activated cells) in the brain microvasculature leads to inflammation-mediated pathology, which is thought to contribute to disease severity both in human and experimental animal models (reviewed in (Schofield and Grau 2005)). For instance, release of TNF- α by inflammatory cells upregulates the expression of ICAM1 to which infected red blood cells bind (Turner et al. 1994, Turner et al. 1998) and platelets enhance cytoadherence of infected erythrocytes (IEs) by bridging the interaction between IEs and endothelial cells in the brain through CD36 binding (Wassmer et al. 2004). In addition, platelet derived microparticles may increase binding of leukocytes and IEs to the endothelium, enhance endothelial permeability and promote apoptosis (reviewed in (Schofield and Grau 2005)). Elevated expression of inflammatory cytokine genes such as TNF- α and IL-1 has been demonstrated in autopsy brain tissues from fatal cases of CM (Brown et al. 1999, Turner et al. 1994). Levels of pro inflammatory cytokines such as TNF- α , IL-1, IL-8, IL-12 and IL-6 measured in serum are also significantly elevated in severe malaria (SM) patients (Lyke et al. 2004, Kwiatkowski et al. 1990, Day et al. 1999) whereas IL-10 and TGF- β are present at low levels (Day et al. 1999). Linking these findings together, presence of mature sequestered parasites leads to recruitment of leukocytes and the ensuing inflammatory environment present in the brain very likely contributes to the pathology of cerebral malaria.

The pathophysiology of severe malarial anemia may in part involve destruction of both infected and uninfected red blood cells, bone marrow suppression of erythropoiesis and altered patterns of inflammatory mediators (reviewed in (Ekvall 2003)). There is a consensus that the clearance of infected red blood cells only plays a minor role towards

severe anemia pathology. Elevated levels of TNF- α and low levels of IL-10 have been reported in young children with severe malarial anemia (Othoro et al. 1999, Kurtzhals et al. 1998). The exact mechanisms of action of cytokines that lead to development of severe anemia are not fully known although *in vitro* experiments suggest that TNF- α (Dufour et al. 2003) and IFN γ (Felli et al. 2005) could play a role in suppression of erythropoiesis. In a study conducted in The Gambia, children presenting with chronic anemia had impaired erythropoiesis and reduced reticulocyte counts (Abdalla and Pasvol 2004) thought to occur due to impaired production of erythropoietin during severe malaria. However, follow up studies showed that the levels of erythropoietin were normal in children with severe anemia (Newton et al. 1997) and more recently *in vitro* experiments demonstrated that decreased responsiveness of erythrocyte precursors in the bone marrow to erythropoietin could explain these findings (Chang, Tam and Stevenson 2004). The clearance of uninfected RBCs by the spleen (Looareesuwan et al. 1987) has been shown to contribute to the development of severe anemia. Impaired RBC deformability is a strong predictor for mortality in children and adults with severe malarial anemia (Dondorp et al. 1997, Dondorp et al. 2002). These cells also undergo intrinsic changes such as oxidation, externalization of phosphatidylserine and increased complement deposition, which targets them for splenic clearance by macrophages (reviewed in (Lamikanra et al. 2007)).

Obstruction of blood flow as a result of parasite sequestration precipitates the development of metabolic acidosis. Tissues undergo anaerobic glycolysis, which leads to accumulation of lactic acid (Day et al. 2000) although lactate production by parasites also augments these levels.

Taken together, the studies above illustrate the pathophysiological diversity of the different malaria syndromes and the complex multifactorial processes that precipitate development of severe disease.

1.5 Management of severe malaria

According to the WHO guidelines, the recommended drug of choice for the treatment of severe falciparum malaria is intravenous (IV) or intramuscular (IM) artesunate (WHO 2010). This policy was informed by the largest trial conducted in Africa, comparing the efficacy of artesunate versus quinine in severely ill children. The study showed a significant reduction in mortality from 10.9% to 8.5% of artesunate versus quinine (Dondorp et al. 2010). For both children and adults, artesunate 2.4 mg/kg of body is administered IV or IM on admission, then at 12 hours and 24 hours. Thereafter, doses are given once a day (WHO 2012). In the absence of parenteral artesunate, artemether or quinine are given as alternative drugs. For patients presenting to hospital with convulsions, treatment with intravenous or rectal benzodiazepines such as diazepam is recommended (WHO 2012). The airways should also be maintained open and breathing support provided if necessary. Use of prophylactic anticonvulsants is not advised following results of a large double-blind placebo trial of 20 mg/kg phenobarbital in Kenyan children that demonstrated doubled mortality rates in children presenting with cerebral malaria who received phenobarbital, possibly due to respiratory arrest (Crawley et al. 2000). In acute renal failure, fluid balance and urinary sodium levels should be checked and patients started on hemofiltration or hemodialysis (Phu et al. 2002). Blood glucose levels should be monitored every four hours particularly in unconscious patients and cases of hypoglycemia (blood glucose levels < 2.2 mmol/l) treated immediately with 0.3–0.5 g/kg body weight of bolus glucose. In areas of high malaria transmission, blood transfusion for severe anemia is recommended for children with hemoglobin levels <5 g/100mL (hematocrit <15%) whereas in areas of low transmission, the threshold is 7 g/100mL (hematocrit <20%) (WHO 2010). However, these recommendations are based on opinions of physicians, as adequate trials to ascertain these practices are lacking. According to the WHO guidelines, patients presenting to

hospital with shock, hypovolemia and respiratory distress particularly with acidotic breathing should receive fluid resuscitation in addition to standard care (WHO 2013). However, results of a large randomized trial in African children comparing fluid resuscitation with albumin or saline against receiving no bolus demonstrated increased mortality in children who received bolus fluids (Maitland et al. 2011) providing strong evidence against this common practice and challenging the existing WHO policy of fluid administration in shock patients (Kiguli et al. 2014). Rapid bolus infusion is contraindicated in individuals of all ages as it could lead to pulmonary oedema. Severe malaria in pregnancy, associated with >50% mortality should be treated with parenteral artesunate over quinine due to recurrent hypoglycaemia associated with quinine. The clinical status of patients with severe malaria deteriorates very rapidly, therefore frequent observation for vital signs, monitoring of blood glucose and hemoglobin levels and parasite counts every 12-24 hours is recommended (WHO 2013).

1.6 Malaria control interventions

The WHO recommends a multifaceted approach for malaria prevention, comprising of (i) vector control using indoor residual spraying (IRS), larval control and use of insecticide treated nets (ITNs), (ii) chemoprevention for the most vulnerable populations by means of intermittent preventive therapy (IPT) and seasonal malaria treatment, (iii) accurate and prompt diagnosis and treatment with highly active artemisinin based combination therapy (WHO 2013).

1.6.1 Vector control strategies

The goal of vector control interventions is to minimize human-vector contact and to lower malaria transmission intensity by reducing the average lifespan of vector

populations (Beier et al. 2008, WHO 2013). In sub-Saharan Africa, scale-up in distribution of ITNs has increased the proportion of the population with access from 10% in 2005 to 42% in 2013 and as a result, many deaths have been averted (WHO 2013). Lengeler *et al* summarized results of individual and cluster randomized trials of ITN usage and showed a protective efficacy of 17% and approximately 50% reduction in incidence of uncomplicated malaria among ITN users (Lengeler 2004). However, although the WHO recommends that all individuals at risk of malaria should sleep under a treated net, current coverage is way below the target required to reduce malaria burden and mortality. In a recent meta analysis of strategies for delivering ITNs at scale for malaria control, the range of ITN usage among children under the age of five was 12% to 94% (Willey et al. 2012).

Approximately forty African countries have adopted indoor residual spraying as a tool for malaria control (WHO 2013). Four general chemical classes of insecticides are recommended for IRS and their uptake in different areas depends on safety profiles, data on insecticide resistance, residual efficacy, cost, community acceptance and types of surfaces to be sprayed. According to the WHO, IRS efficacy against malaria-attributable mortality and reducing malaria transmission in targeted communities can only be achieved if >80% of houses and shelters are treated (WHO 2013). Selection pressure due to widespread use of pyrethroids for agriculture and treatment of bed nets has contributed to emergence of resistance (Ranson et al. 2011, Brouqui, Parola and Raoult 2012, Himeidan et al. 2011). Larval treatment which involves regular treatment of water bodies with a biological or chemical compound to reduce the number of mosquito larvae is difficult to achieve in rural settings since mosquito breeding sites are often temporary, widely spaced and difficult to enumerate. Larviciding is therefore mostly feasible in urban areas where the breeding sites are few, easy to find and permanent.

1.6.2 Chemoprevention and chemoprophylaxis

In areas of moderate to high malaria transmission, the WHO recommends administration of a full course of sulphadoxine-pyrimethamine (SP) to all pregnant women at each scheduled antenatal visit after the first trimester (WHO 2004). Each dose should be administered at least one month apart up to the time of delivery. This approach has also been extended to infants (intermittent preventive treatment in infants–IPTi) through the routine expanded immunization programme. Regardless of the presence or absence of parasitemia, infants receive three doses of SP in total, each given along with the second and third doses of DPT and measles vaccines. Pooled data obtained from six randomized trials of IPTi with SP demonstrated partial protection against uncomplicated malaria (30%), anemia (21%) and hospital admissions with malaria parasitemia (38%), but no effect on mortality (Aponte et al. 2009). The WHO also recommends seasonal malaria chemoprevention (SMC) to all children aged 3-59 months in areas of highly seasonal malaria transmission (WHO 2013). Children receive four doses of amodiaquine plus SP, each administered monthly from the start of the malaria transmission season. Results from a meta analysis of twelve studies showed an overall protective efficacy of 82% during the malaria transmission season and 57% against all-cause mortality (Wilson 2011), suggesting that SMC is a highly effective control approach in areas with markedly seasonal malaria transmission.

1.7 Malaria vaccines

The unprecedented success of vaccines against polio, tetanus, diphtheria and the successful eradication of small pox and rinderpest demonstrates without doubt the potential of this approach in alleviating the burden of infectious diseases (Andre et al. 2008, Levine and Robins-Browne 2009). Of note however, is the unavailability of vaccines against parasitic infections including malaria, despite more than 30 years of

ongoing efforts to develop one. More than 40 vaccine-testing projects are currently underway (reviewed in (Schwartz et al. 2012)) and there are renewed efforts to identify additional candidates from the over 5,200 parasite proteins (Gardner et al. 2002). Vaccine targets are based on the pre-erythrocytic, erythrocytic and sexual stages of parasite development in the human host.

1.7.1 Pre-erythrocytic stage vaccines

Experiments conducted in the 1970s demonstrated that vaccination with radiation-attenuated sporozoites could induce sterile protection in humans (Clyde et al. 1973) and animals (Nussenzweig et al. 1967, Gwadz et al. 1979). Despite this demonstration, there has been relatively slow progress towards advancement of this vaccination approach, until recently, owing to i) the requirement of a high dose of irradiated sporozoites (>10,000) to induce sterile protection that is difficult to achieve technically, in large quantities and to good manufacturing practice (GMP) standards and ii) the number of mosquito bites required to inoculate such a high sporozoite dose (5-19 biting sessions). The challenge of producing large doses of aseptic and purified sporozoites (*PfSPZ*) has been resolved recently (Hoffman et al. 2010, Epstein et al. 2011) and in a subsequent clinical trial, Epstein *et al.* demonstrated that the route of administration of viable *PfSPZs* was crucial in achieving protective efficacy. Volunteers who received the dose via subcutaneous or intradermal injection using a needle and syringe mounted suboptimal immune responses and achieved inadequate protection (Epstein et al. 2011) in comparison to individuals who were immunized with non-irradiated sporozoites via mosquito bites followed by a prophylactic regimen of chloroquine (Roestenberg et al. 2011). This could be attributed to inadequate antigen presentation since most of the sporozoites are dead after cryopreservation (Chakravarty et al. 2007).

Given the challenges of deploying a vaccine in the field using mosquito bites,

determination of an optimal route of administration of the sporozoites is crucial and in experiments conducted in non-human primate models, intravenous administration of *Pf*SPZ elicited potent and durable sporozoite-specific T cell responses (Epstein et al. 2011). Recently in a human trial, repeated intravenous administration of *Pf*SPZ using needle and syringe elicited high level, dose-dependent protection against controlled human malaria infection (CHMI) (Seder et al. 2013) suggesting that this inoculation approach may be effective in future.

Other research groups have focused on development of subunit sporozoite vaccines and currently the candidates under clinical development are CSP, LSA-1, Exp-1 and SSP2/TRAP. The most advanced malaria vaccine, the RTS,S vaccine from GlaxoSmithKline (GSK), is based on the NANP central repeats of the circumsporozoite protein (CSP) plus T-helper epitopes from the C-terminal region fused to hepatitis B surface antigen (RTS). This component is co-expressed with more free hepatitis B surface antigen in a 1:4 ratio to form the RTS,S virus-like particle (reviewed in (Cohen et al. 2010)). RTS,S antigen formulated with the liposome-based (AS01) or oil-in-water based (AS02) adjuvant systems together with monophosphoryl lipid A (Baldrige and Crane 1999) and QS21 (Kensil, Wu and Soltysik 1995) as immunostimulants have been extensively evaluated. Comparative studies of RTS,S/AS01 and RTS,S/AS02 have demonstrated superior anti-CS responses and efficacy with RTS,S/AS01 when three doses are administered both in adults (Kester et al. 2009, Polhemus et al. 2009) and children (Agnandji et al. 2011). In Phase III trials of RTS, S/AS01 in African children, the overall efficacy against clinical episodes of malaria was 30.1% (Agnandji et al. 2012) and 50.4% (Agnandji et al. 2011) in infants aged 6 – 12 weeks and 5 – 17 months, respectively. A follow-up study also showed that the RTS, S vaccine does not induce durable protection against clinical episodes of malaria over a four-year follow-up period (Olotu et al. 2013). Substantial age-dependent differences in efficacy were

observed possibly due to interference by co-administered expanded program on immunization (EPI) vaccines, presence of maternal antibodies or inherently lower immune responses in infants compared to older children. Admittedly, the protective efficacy of RTS,S falls short of the goals of the original malaria vaccine technology roadmap which is to develop and license a first-generation malaria vaccine that has a protective efficacy of greater than 50% against severe malaria and death and lasts longer than one year, by 2015 (WHO 2013). Therefore, improvements on the RTS,S vaccine, for instance, by combining it with antigens targeting other stages of the parasite life cycle may confer better protection.

Although contemporary approaches have assessed responses to single antigens, formulations comprising of multiple pre-erythrocytic epitopes or target antigens are also being evaluated. For instance, the multi-epitope TRAP (consisting of CD4+ and CD8+ T cell epitopes from six antigens; LSA1, CSP, STARP, LSA3, Exp1 and TRAP) fused to the TRAP antigen (Moorthy et al. 2003) and the DNA vaccine polyepitope DNAEPI 1300 (consisting of CSP, SSP2/TRAP, LSA-1, Exp1) administered through electroporation. Vaccination with the ChAd63-MVA-TRAP prime-boost regimen demonstrated a highly favorable safety profile and robust immunogenicity in both malaria naïve volunteers (Ewer et al. 2013) and malaria exposed Gambian and Kenyan adults (Ogwang et al. 2013).

1.7.2 Erythrocytic vaccines

Seminal studies demonstrating marked reduction of parasitemia and resolution of clinical symptoms of malaria in children receiving purified IgG obtained from malaria exposed adults (Cohen, Mc and Carrington 1961) have provided the impetus for development of vaccines against the blood-stage of infection. The specificities of these antibodies are not completely known but antigens expressed at the merozoite stage and

those inserted on the surface of the infected red blood cell are attractive options due to their accessibility to the immune system. At present, vaccines based on antigens exported to the merozoite surface during invasion such as AMA1 (Thera et al. 2010, Thera et al. 2011), EBA-175 region II (El Sahly et al. 2010) and those expressed at the surface of the merozoite such as MSP-1 (Ogutu et al. 2009), MSP-3 (Belard et al. 2011), SERA5 (Horii et al. 2010), GLURP (Belard et al. 2011) and MSP-2 (Genton et al. 2002) are being evaluated in clinical trials. AMA1 and MSP1 proteins are the most advanced blood-stage candidates, tested using various formulations such as AMA1-C1 (3D7 and FVO) alhydrogel with or without CpG (Sagara et al. 2009, Dicko et al. 2008, Ellis et al. 2009), FMP2.1/AS02A or AS01B (Spring et al. 2009, Thera et al. 2008, Thera et al. 2010), FMP1/AS02A (MSP1₄₂) (Stoute et al. 2007, Ogutu et al. 2009, Withers et al. 2006), ChAd63-MVA/MSP1 (Sheehy et al. 2011) and combining MSP1 and AMA1 (Malkin et al. 2008, Sheehy et al. 2012b). Disappointingly, neither AMA1 (Thera et al. 2011) nor MSP1 (Ogutu et al. 2009) has demonstrated adequate clinical efficacy when tested in paediatric populations in Africa, possibly due to the extensive antigenic polymorphism exhibited by these antigens and the induction of predominantly low to modest antibody titres. The focus has also been extended to combinations of blood-stage antigens or blood-stage antigens together with other developmental stages. For instance, the combination B vaccine comprising of RESA, MSP-1 and MSP-2 (3D7) formulated with an oil-based adjuvant, Montanide ISA 720 conferred a 62% reduction in parasite density in Papua New Guinean children who did not receive SP drugs, but increased carriage with parasites containing the alternate MSP-2 allele (Genton et al. 2002). However, testing of this vaccine was discontinued. The GMZ2 vaccine combining the MSP-3 and GLURP antigens with aluminum hydroxide was safe, well tolerated and immunogenic when tested in Gabonese children (Belard et al. 2011). Thus far, there is no indication that these combination blood-stage vaccines confer superior

protection compared to single antigen vaccines although evidence from immuno-epidemiological studies increasingly shows the importance of the breadth of responses in terms of the number of antigens an individual responds to, in inducing higher protective natural immunity compared to responses to single antigens (Osier et al. 2008, John et al. 2005).

Antigens expressed on the surface of the infected red blood cell are plausible but challenging vaccine targets to develop due to their temporal switching of gene expression patterns and high molecular mass (Fried et al. 2013). However, identification of conserved domains or domain combinations within the widely studied PfEMP1 protein that are immunogenic and induce broadly cross neutralizing antibodies could circumvent this difficulty. Efforts to identify conserved regions (Avril et al. 2010), highly immunogenic domains and domain combinations (Saveria et al. 2013, Fried et al. 2013) are currently underway for var2CSA, a PfEMP1 variant associated with pregnancy malaria by mediating placental sequestration.

1.7.3 Transmission blocking vaccines

Transmission blocking vaccines (TBV) are designed to block development of infectious sporozoites in the mosquito vector but not to protect the vaccinated individual from infection. Such vaccines target antigens expressed on gametocytes, zygotes or ookinetes. The demonstration that individuals living in malaria endemic areas acquire potent transmission-blocking antibodies (Bousema et al. 2006) forms the basis for development of transmission-blocking vaccines, a tool that could contribute towards malaria elimination and eradication.

The three leading TBV targets for *P. falciparum* induce antibody responses against the parasite antigens Pfs230, Pfs48/45 and Pfs25. Pfs48/45 and Pfs230 are expressed on the surface of gametocytes and play a role in fertilization of macrogametes by

microgametes (Vermeulen et al. 1985, Rener et al. 1983, Kumar and Carter 1984), whereas Pfs25 is expressed on the surface of ookinetes (Pradel 2007, Vermeulen et al. 1985). Both Pfs48/45 and Pfs230 are attractive vaccine candidates because they are expressed in the human host and antibody responses to these antigens could potentially be boosted by natural infection unlike Pfs25. A Phase 1 human trial using a recombinant Pfs25H formulated with Montanide ISA51 demonstrated that human Pfs25 specific antibodies block parasite infectivity to mosquitoes (Wu et al. 2008), although some subjects reported serious adverse reactions to the vaccine.

Evidence from mathematical modeling studies suggest that an effective TBV would need to induce very high antibody levels (Saul 2008) and such a vaccine would only be efficacious in areas of high malaria transmission intensity if administered together with other interventions such as bednets (Carter et al. 2000). However, the combination of a TBV with other candidates such as blood-stage or pre-erythrocytic antigens would be an ideal vaccination strategy because, if effective, it would reduce transmission and also provide protection against infection or disease to the vaccinated individuals.

There is renewed interest to apply systematic approaches for identifying and prioritizing antigens to put forward for vaccine development. These include i) use of state-of-the art techniques such as functional genomics to screen novel antigens and ii) accelerating the timelines for clinical development by including controlled human malaria infections studies. Towards this end, the full genome sequence of *P. falciparum* genes has increased the range of targets available for study (Gardner et al. 2002) coupled with high throughput screening approaches involving assembly of multiple proteins on arrays which are used to screen specific responses (Doolan et al. 2008, Crompton et al. 2010a, Trieu et al. 2011). In addition, development of eukaryotic expression systems to express full-length proteins preserving their structural conformation and function has enabled

high throughput testing of protein functions such as protein-receptor interactions (Crosnier et al. 2013, Crosnier et al. 2011, Bartholdson et al. 2013). Such platforms have led to the identification of *PfRh5* as a promising second-generation blood-stage vaccine candidate that has been shown to induce strain-transcending neutralizing antibodies in pre-clinical studies (Douglas et al. 2011, Reddy et al. 2014).

1.8 Naturally-acquired immunity to malaria

Individuals living in malaria endemic areas who do not succumb to severe malaria during the first few years of life remain susceptible to clinical episodes of malaria to which they acquire immunity during early adulthood. Therefore, adults rarely if ever suffer clinical attacks but often harbor asymptomatic infections (reviewed in (Marsh and Kinyanjui 2006, Langhorne et al. 2008)). In the early 20th century, blood-stage parasite inoculations were used to treat neurosyphilis patients by induction of high fevers that killed the *Treponema pallidum* spirochetes. Re-analysis of patient records by Collins and Jeffery in the 1990s showed a reduction of the frequency of fever, parasitemia and gametocyte carriage in patients exposed to secondary infections, suggesting that immunity against malaria could be induced in humans (Collins and Jeffery 1999a).

The key features of naturally-acquired immunity (NAI) are, first, it develops after frequent exposure to infections, evidenced by the high burden of morbidity and mortality to severe life-threatening episodes in relatively naïve young children having little exposure (Marsh and Kinyanjui 2006, Langhorne et al. 2008) and in malaria naïve travelers. By the age of five, children acquire immunity to severe episodes but remain susceptible to clinical episodes of malaria until early adulthood. Modeling studies have suggested that immunity to severe non-cerebral episodes may develop after one or two infections (Gupta et al. 1999). Second, NAI is not sterile, demonstrated by the frequent parasitization seen in older children and adults living in an endemic area (Marsh and

Kinyanjui 2006, Tran et al. 2013) and the ability to maintain chronic asymptomatic infections in some individuals. In their study, Tran *et al* followed up 251 children and adults aged 4 – 25 years over a 6 month period for development of clinical episodes of malaria and PCR confirmed infections and revealed no age-related differences in the risk of infection (Tran et al. 2013). A limitation of this study was the narrow age range of adults selected. One may argue that individuals aged 25 years (the upper age limit in this study) are in fact very young adults and their findings would have been more conclusive had they showed the risk of infection in relatively older individuals. Maintenance of chronic infections was also demonstrated in neurosyphilis patients inoculated with *P. falciparum* who had persistent low-grade asymptomatic infections over an extended period after recovery from malaria symptoms accompanied by successive waves of parasitemia (Collins and Jeffery 1999b).

Third, NAI diminishes upon interruption of exposure (Matteelli et al. 1999, Jelinek et al. 2002). In the highlands of Madagascar, DDT was successfully used in the 1950s to eradicate malaria. However, a sudden resurgence occurred in 1980, which resulted in epidemics that killed over 40,000 adults and children (Mouchet et al. 1997, Deloron and Chougnet 1992). Of note, adults aged 40 years and above who had spent their childhood years under continuous malaria exposure were more protected than younger children and they also had stronger humoral responses suggesting some degree of immunological memory (Deloron and Chougnet 1992). This latter finding is corroborated by observational studies demonstrating that some degree of immunity to clinical malaria is achieved in immigrants compared to malaria naïve adults (Bouchaud et al. 2005, Mascarello et al. 2008). In the study by Bouchaud *et al*, African immigrants who had lived in Europe for at least four years had lower parasite densities, faster parasite clearance times and higher antibody levels compared to Europeans, following short visits to sub-Saharan Africa (Bouchaud et al. 2005). Therefore, although malaria

immunity wanes in the absence of continuous exposure, this is only partial. Such findings have important implications in the current era of a move towards malaria eradication and the observed decline in malaria transmission intensity in some areas. History has taught us that if current elimination and eradication efforts are successful but not long-term and sustainable, a population of susceptible individuals would emerge leading to future epidemic attacks.

The slow acquisition of immunity to malaria can be attributed in part to the need to accumulate responses against multiple independent parasite strains. In classical experiments done by Brown and Brown in the 1960s, monkeys were resistant to reinoculation with homologous parasite strains but were susceptible to clinical episodes when challenged with a heterologous strain (Brown and Brown 1965). In line with these findings, experiments done in neurosyphilis patients revealed that individuals were resistant to infection with a homologous parasite strain but susceptible to a different strain (Collins and Jeffery 1999a). Indeed, antigens expressed on the infected red blood cell surface have been shown to vary antigenically (reviewed in (Scherf, Lopez-Rubio and Riviere 2008)). This is further exacerbated by the presence of extensive genetic diversity in many *P. falciparum* antigens (Takala et al. 2009) to which individuals induce variant specific (Marsh and Howard 1986, Bull et al. 1998) and allele-specific (Osier et al. 2007, Osier et al. 2010b, Osier et al. 2010a, Cortes et al. 2005, Genton et al. 2002) responses. Adults and older children have a broad repertoire of responses to the different variants, which is associated with natural acquisition of immunity to malaria (Marsh and Howard 1986, Bull et al. 1998).

The rate at which naturally-acquired immunity develops in malaria endemic areas is thus dependent on the degree of exposure, with immunity developing more rapidly in areas of high malaria transmission intensity compared to areas of low malaria transmission, with children bearing the brunt of disease.

In summary, the clear picture that emerges from epidemiological observations is that development of immunity to malaria is dependent on both age and exposure, two factors that are difficult to disentangle given that increasing age results in increased cumulative exposure. However, in cross-sectional surveys of non-immune Indonesian transmigrants, children and adults had a similar incidence of parasitemia after three months of residence in a holo-endemic area but when prospectively examined after 20 months, the adults had less frequent and less severe infections compared to children (Baird 1995, Baird et al. 1993). These observations suggest that acquisition of immunity is in part determined by intrinsic age-related factors independent of the degree of exposure to infection (Doolan, Dobano and Baird 2009).

1.8.1 The role of antibody and cellular responses in naturally-acquired immunity to malaria

Passive transfer studies conducted in 1960 by Cohen *et al.* demonstrated that immunoglobulins purified from immune adults could be successfully used to treat children with episodes of malaria (Cohen et al. 1961). Consistent with these findings, IgG from African adults administered to Thai children led to resolution of clinical symptoms and clearance of parasites (Sabchareon et al. 1991). These findings suggest that naturally-acquired antibodies, particularly of the IgG subclass play an important role in protection against malaria. The underlying mechanisms of protection, why they are acquired only after repeated infections and the plausible targets from over 5,400 parasite proteins (Gardner et al. 2002) are incompletely understood.

The potential targets of antibodies include antigens exposed to the immune system during the merozoite and mature stages of asexual development. Prospective longitudinal studies measuring responses to specific antigens at the start of a malaria transmission season and following individuals through the rainy season for development

of malaria have been pivotal in objectively identifying protective targets of naturally-acquired antibodies. Out of a total of 33 studies included in a meta-analysis of the risk of developing clinical episodes of malaria in the presence of antibodies to approximately 10 *P. falciparum* merozoite antigens, only antibodies directed to the C-terminus of MSP-3 and MSP-1₁₉ and to a somewhat lesser extent, AMA1 and GLURP were consistently associated with protection across all studies (Fowkes et al. 2010). This is not surprising given the differences in methodologies between studies such as choice and preparation of antigens, antibody quantification methods, follow up time for clinical disease and definitions of clinical endpoints. Standardization of methodologies for measurement and reporting of protective efficacies of specific antibodies across settings is important in order to accurately determine the contribution of antibodies to blood stage targets in naturally-acquired immunity to malaria.

Antibodies are thought to function by blocking merozoite dispersal after schizont rupture (Green et al. 1981), blocking merozoite invasion into red blood cells (Blackman et al. 1990, Kocken et al. 2002), opsonizing merozoites for phagocytosis (Hill et al. 2012), antibody-dependent cellular inhibition (ADCI) by monocytes (Bouharoun-Tayoun et al. 1995) or antibody-dependent respiratory burst (ADRB) by neutrophils (Joos et al. 2010).

Some immuno-epidemiological studies investigating the association between antibodies expressed on the surface of the infected erythrocyte and risk of malaria have demonstrated that PfEMP1 is the major target (Chan et al. 2012), and antibodies to some PfEMP1 variants are associated with a reduced risk of developing malaria (Marsh et al. 1989, Chan et al. 2012, Giha et al. 2000, Mackintosh et al. 2008b, Dodo et al. 2001) albeit inconsistently (Bull et al. 2002). Several studies have shown that children rarely have antibodies to the variant surface antigens (VSAs) expressed by the infecting isolates but these are quickly acquired after infection, and subsequent disease episodes

will be caused by parasites with VSA types that are not already recognized by the individual (Marsh and Howard 1986, Bull et al. 1998, Ofori et al. 2002). Although PfEMP1 undergoes rapid antigenic switching suggesting that the acquisition of a broad repertoire of antibodies is necessary to mediate immunity, there is evidence that cross-reactive antibodies are present in immune individuals (Marsh and Howard 1986, Bull et al. 2000, Chattopadhyay et al. 2003), which may confer broad protection against a diverse number of phenotypes (Aguilar et al. 1992, Nielsen et al. 2004). Furthermore, some studies have shown that only limited subsets of common PfEMP1 variants are associated with severe disease (Rottmann et al. 2006, Jensen et al. 2004) and are more frequently recognized by sera from malaria-exposed individuals, including young children, than parasite antigens from older children with mild malaria (Bull et al. 2000, Nielsen et al. 2002). These studies taken together beg the question whether immunity to severe disease is rapidly acquired (Gupta et al. 1999) because only a limited number of variants are associated with severe disease and if so, narrowing down on conserved, cross-reactive epitopes or the small proportion of PfEMP1 variants associated with severe disease may improve our understanding of the contribution of anti-PfEMP1 antibodies in mediating naturally-acquired immunity. Antibodies directed to the infected erythrocyte surface have also been shown to mediate their function by opsonizing infected erythrocytes for uptake by monocytes (Celada, Cruchaud and Perrin 1982, Chan et al. 2012, Ghumra et al. 2011), preventing/disrupting rosette formation (Carlson et al. 1990, Barragan et al. 1998) and inhibiting parasite sequestration on receptors expressed on endothelial cells (Udeinya et al. 1981).

Antigens expressed at the pre-erythrocytic stage are targets of antibodies that block invasion of hepatocytes. Anti-sporozoite antibodies are detectable in sera collected from individuals living in malaria endemic areas (Nardin et al. 1979, Druilhe et al. 1986) predominantly against CSP, the major antigen expressed on the surface of sporozoites.

CSP (Del Giudice et al. 1987, Marsh et al. 1988), LSA (John et al. 2003) and TRAP (Scarselli et al. 1993) antibodies are acquired in an age-dependent manner but have been inconsistently associated with protection against infection or clinical episodes of malaria. Some studies report a decreased risk of infection (John et al. 2005) or clinical episodes of malaria (John et al. 2008, Nebie et al. 2008b, Migot-Nabias et al. 2000) whereas others have reported lack of protection (Kitua et al. 1999, John et al. 2003). Individuals who mount high antibody levels against multiple pre-erythrocytic stage antigens (John et al. 2008, John et al. 2005) have a reduced risk of developing a clinical episode of malaria compared to those who make responses to single antigens.

Both animal (Weiss et al. 1988, Schmidt et al. 2008, Rodrigues et al. 1997, Tsuji et al. 1990, Oliveira et al. 2008) and experimental human challenge models (Roestenberg et al. 2011, Bijker et al. 2014, Pombo et al. 2002) have demonstrated an important role for both CD4⁺ and CD8⁺ T cells in protective immunity against malaria. Although the T cell correlates of immunity to malaria are not completely understood, there is a consensus that the timing and fine balance between pro-and anti-inflammatory cytokine secretion is important for the successful resolution of an infection (Riley et al. 2006). Some field-based studies have shown that IFN-gamma induced by T-cells against liver-stage (Reece et al. 2004, Luty et al. 1999, Todryk et al. 2008) and blood-stage antigens (Robinson et al. 2009, Luty et al. 1999, Moormann et al. 2013, D'Ombrian et al. 2008, McCall and Sauerwein 2010, Dodoo et al. 2002) is associated with resistance to reinfection or clinical episodes of malaria. IFN γ production by Th1 cells and other lymphocytes may therefore be a potential mediator of protective immunity.

A population of IFN γ /IL-10 co-producing CD4⁺ T cells has been recently defined in children living under intense malaria exposure (Jagannathan et al. 2014) and those encountering re-exposure to *P. falciparum* parasites after a recent febrile episode of malaria (Portugal et al. 2014). These cells have been described as “self-regulating” Th1

effector cells (O'Garra and Vieira 2007) acting to limit excessive production of pro-inflammatory mediators while enhancing effector mechanisms that control parasite replication. Failure to detect a significant number of circulating IFN γ /IL-10 co-producing CD4 $^{+}$ cells at the time of acute infection in a separate study conducted in an area of moderate malaria transmission suggests that these cells are produced in an exposure-dependent manner (Gitau et al. 2012). Furthermore, the presence of pre-existing IFN γ /IL-10 co-producing CD4 $^{+}$ cells was not associated with protection from future episodes of malaria (Jagannathan et al. 2014) suggesting that they may not play an important role in protective immunity, but these findings remain to be confirmed in other settings.

1.8.2 Antibodies to blood-stage antigens and risk of severe malaria

The observation that children living in malaria endemic areas who survive the first five years of life develop lifelong immunity to severe malaria but remain susceptible to clinical episodes of malaria suggests that the dominant factors or targets conferring protection against severe illness may be different from those mediating immunity to clinical episodes of malaria or parasitization, which is seen in older children and adults. Nonetheless, there is a potential overlap between the mechanisms and targets that mediate the different phases of immunity because the ability to limit parasite multiplication to very low levels is crucial in preventing the parasites from overwhelming an individual and leading to development of clinical symptoms. There are very limited epidemiological data defining the targets and mechanisms of protection against severe malaria owing to the fact that such studies require recruitment and follow-up of a large number of children for the relatively rare outcome of severe malaria. For instance, in a study conducted at the Kenyan coast, among 4,783 children

recruited at the start of a malaria transmission and followed up over a period of 8 months during two transmission seasons, only 21 cases of severe malaria were identified (Bull et al. 1998, Ndungu et al. 2002). In this section of the thesis, I review the existing literature on the role of antibodies in protection against severe malaria and highlight the gaps in knowledge that form the basis of this thesis.

First, studies designed to investigate the importance of antibodies to specific targets have been largely hospital-based, comparing antibody responses in children admitted to hospital with severe malaria against healthy or uncomplicated malaria controls (Table 1). Responses measured at the time of acute infection comprise those generated in response to the current infection as well as pre-existing ones, which makes it difficult to infer a causal relationship between antibodies and the outcome of severe malaria. Prospective studies in which pre-existing antibodies are analyzed in relation to the subsequent risk of malaria are preferable but thus far, such studies have been few, small in size, inadequate in the definition of severity and only evaluated a limited number of antigens (Bull et al. 2002, Ndungu et al. 2002, Osier et al. 2008, Bull et al. 1998). In the study by Ndungu *et al* and Bull *et al*, only 21 well-defined severe malaria cases were examined and antibodies to non-specific antigens contained in a crude schizont extract (Ndungu et al. 2002) and those to the surface of three infecting parasite isolates (Bull et al. 2002) were measured. In these studies, IgG1 against crude schizont extract was associated with a decreased risk of developing severe malaria (Ndungu et al. 2002) whereas agglutinating antibodies directed to the infected red blood cell surface were not associated with a reduced risk of developing severe malaria (Bull et al. 2002).

In the studies by Bull *et al* and Osier *et al*, severe malaria was defined as malaria severe enough to warrant admission to hospital (Bull et al. 1998, Osier et al. 2008) with no indication of whether specific severe malaria symptoms were present and in what proportion of children. The study by Osier *et al*, demonstrated that the antibody

concentration and responses to combinations of *P. falciparum* merozoite antigens could be more important in protection against admission to hospital with malaria (Osier et al. 2008), but this remains to be validated for well-defined severe malaria as a clinical endpoint.

Second, hospital-based studies have often reported conflicting results with some studies showing no differences in antibody levels between severe malaria cases and mild malaria cases (Erunkulu et al. 1992, Al-Yaman et al. 1997), whereas others report significantly higher antibody levels in mild malaria cases compared to severe malaria cases (Okech et al. 2006, Perraut et al. 2005a). Conversely, others have reported higher antibody levels in severe malaria cases compared to mild malaria cases (Dobano et al. 2008). The inconsistencies in findings have arisen partly due to lack of standardized methodologies for measuring and reporting data across studies. Table 1 below summarises the findings from 18 hospital-based studies comparing antibody responses against several *P. falciparum* merozoite and variant surface antigens in severe malaria cases, uncomplicated malaria cases and healthy controls. The key differences identified in these studies that lead to inconsistent findings include i) varying definitions of severe malaria with some studies either focusing on the specific severe malaria manifestations of cerebral malaria, hyperparasitemia, hypotension, severe anemia and respiratory distress and others considering any of the above symptoms; ii) the choice of controls to compare against ranging between healthy controls, uncomplicated malaria controls, controls with asymptomatic infections and other unrelated severe illnesses; iii) differences in antigens selected for study ranging between conserved and polymorphic merozoite antigens, crude parasite lysate and variant surface antigens; iv) differences in age inclusion ranging from children to adults depending on transmission intensity; and v) the antibody response measured ranging from total IgG, IgM to IgG subclasses. Therefore, despite the large body of literature from hospital-based studies, the evidence

for a role of antibodies and their targets in protection against severe malaria remains inconclusive.

Table 1: A summary of hospital-based case-control studies examining the association between antibodies to *P. falciparum* blood-stage antigens and protection against severe malaria.

Author, year	Country	Age group	Study design	Study population	Antigens tested	Findings
(Okech et al. 2006)	Uganda	6-59 months	Matched-case control (matched on age, sex, geographic location)	SM ¹ (N=103): Fever, any SM syndrome and parasitemia >10,000p/μl UM ² (N=102): Parasitemia <5,000p/μl with or without fever	Serine Repeat Antigen 5 (SE36, N-terminus and SE50A, C-terminus) Schizont extract	Prevalence and median anti-SE36 and anti-schizont lysate IgG levels were higher in UM compared to SM children.
(Dobano et al. 2008)	Malawi	Mean age 3 years	Case-control study	CM ³ (N=126): BCS<3 and any parasitemia SMA ⁴ (N=59): Hb<5g/dL or hematocrit <15% and any parasitemia UM (N=84): patients discharged with a final diagnosis of malaria, children screened for enrolment in malaria drug studies	MSP-1 block 2 (RO33, MAD20 and K1) MSP-1 ₁₉ , MSP-1 ₄₂ MSP-2 (FC27 and 3D7), C-terminus (K1) AMA1 (3D7) full ectodomain RAP-1	Prevalence and mean IgG levels against all antigens tested were higher in CM patients compared to SMA and UM patients. Prevalence and mean IgG levels are lower in the SMA patients compared to the UM patients.

(Iriemenam et al. 2009)	Sudan	6 months to 64 years	Matched case-control (healthy controls matched on age, sex and residence to CM and SMA patients)	CM (N=118): Parasitemia and unarousable coma lasting >30mins after a seizure SMA (N=58): Parasitemia, Hb<5g/dL or HCT <15% UM (N=230): Parasitemia, fever, headache HC ⁵ (N=139): No detectable parasites.	AMA1 (3D7) full ectodomain MSP-2 (FC27 and 3D7) MSP-3 (Long synthetic peptide) GLURP-R0 and GLURP-R2 Measured total IgG and subclasses	Predominantly IgG1 and IgG3 responses against all antigens tested. AMA1 antibodies were predominantly of the IgG1 subclass. These were higher in the UM group compared to the SM group and HC in all age groups. GLURP-R0 and R2 IgG1 and IgG3 levels were higher in the UM group compared to the SM group. Levels were also higher in the SM group compared to the HC. MSP-2 (FC27) and MSP-3 IgG3 antibodies were higher in the UM group compared to the SM and HC groups in the >5yr age group.
(TM et al. 2008)	Sudan	0 – 45 years	Matched case-control (UM and HC controls matched on age, sex, residence and date of inclusion into	SM (N=109) Patients satisfying the WHO criteria ⁶ for SM. UM (N= 114): Fever and parasitemia. HC (N=117)	MSP1 ₁₉ MSP2 (A) GF/88 MSP2 (B) T9/96	Antibody prevalence was significantly higher in the UM group than in the SM group for all antigens tested. Antibody prevalence was higher in the SM group compared to the

	study, to SM cases)				healthy controls for all antigens tested.
(de Souza et al. 2002)	Gambia	6months – 15 years	Case-control study	SMA (N=22) according to WHO definition	GPI
				CM (N=84) according to WHO definition	Mean antibody levels were significantly higher in children with malaria compared to non-malaria control group.
				UM (N=90)	No difference in prevalence or mean anti-GPI antibody levels between UM, SMA and CM groups.
				Severe nonmalarial illnesses (n=65)	
				Mild nonmalarial illness (n=80)	
(Perraut et al. 2005a)	Senegal	Case-control study	<u>Group 1</u> >13 years <u>Group 2</u> 2-63 years	<u>Group 1</u> Sampled in 1998 – 1999 CM (N=35) UM (N=35) <u>Group 2</u> Sampled in 2000-2001 CM (N=52) Fatal CM (N=18)	<u>Group 1</u> UM patients had higher anti-GPI antibody prevalence and mean levels compared to CM patients. There was no difference in levels of anti- MSP1 ₁₉ antibodies between the two groups <u>Group 2</u> Mean anti-GPI antibody levels were significantly higher in the UM group compared to the CM

					UM (N=30)	and HC group.
					HC (N=47)	There was no difference in levels of anti- MSP1 ₁₉ antibodies between the two groups. HC had lower MSP1 ₁₉ antibodies.
(Erunkulu et al. 1992)	Gambia	0-8 years	Matched case-control study (Matching done on age, ethnic group and residence)	SM (N=244): Modified GCS <3 or Hb<5g/dL	Unfractionated schizont antigen	Mean antibody levels are higher in children with malaria than in children with other illnesses.
				UM (N= 136): Fever and parasitemia	CSP (NANP) ₄₀	Mean Ab levels to all antigens tested in children with severe and mild malaria were similar (adjusted for age and residence.)
				Severe non-malarial controls (N= 93) Admission with severe infections other than malaria	P/155/RESA	
				Mild controls (N=76): mild infections other than malaria treated at the OPD	MSP1	No difference in mean Ab levels between CM and SMA were observed.
(Luty et al. 2000)	Gabon	Mean age 44 months	Matched case-control study (Matched on age, gender and provenance)	SM (N=100): Hb <5g/dL or hematocrit <15% and/or hyperparasitemia (>250,000p/ul)	CSP (NANP) ₆	No difference in mean IgG and IgM antibody levels between CM and UM.
				UM (N=100): parasitemia (1,000-50,000 p /ul), Hb>8g/dL, glycemia >50mg/dL and no	Schizont extract	At admission, the median IgG and IgM antibody levels to NANP and schizont were similar between the cases and controls.
						Significantly lower IgG levels against NANP in the SMA group compared to the non-

signs of SM.					
SMA and UM group. No difference between the three groups in IgG responses to schizont extract					
(Al-Yaman et al. 1997)	Papua New Guinea	0-17 years	Matched case-control study (Matched on age, time of disease onset and residence)	CM (n=97): Coma, BCS<3, parasitemia UM (n=146): Fever, parasitemia, Hb>5g/dL	MSA-1-C-terminal MSA-2 (FC27 and 3D7) RESA Schizont extract
No significant difference in the mean antibody concentration and prevalence in the CM and UM patients for all antigens tested.					
(Tangteerawatana et al. 2007)	Thailand	13-67 years		SM (N=110): WHO criteria for SM. UM (N=169): Fever, parasitemia	Schizont extract
Mean IgG, IgG2 and IgG3 levels were significantly higher in the UM group compared to the SM group. IgE levels were also higher in the UM group compared to the SM group but the difference was not significant.					
In a multivariate logistic regression analysis, lower levels of IgG3 were associated with increased risk of SM.					
(Tharavanij et al. 1984)	Thailand	6-70 years		Uncomplicated CM (N=47): unarousable coma plus	Merozoite extract
By IHA and IFA, patients with CM (complicated & uncomplicated) had Ab levels					

				parasitemia	Schizont extract	similar to the UM group. By IHA, patients with complicated CM had significantly lower antibody levels compared to uncomplicated CM and UM cases.
				Complicated CM (N=53): coma plus other complications e.g. hyperparasitemia, coma>24hrs, jaundice, renal failure, hypotension, pulmonary edema. UM (N=108) HC (N=100)		
(Tebo et al. 2002)	Gabon	6months – 11 years	Matched case-control study (Matched on age, gender and provenance)	SM (N=95): Hb<5g/dL and/or hyperparasitemia(>250,000p/μl) with or without other signs of severe malaria UM (N=95): parasitemia (1,000-50,000p/μl), Hb>8g/dL, glycemia >50mg/dL	<i>P. falciparum</i> infected erythrocytes Cys007 – obtained from a 38month old child with SM Cys028- obtained from a 12month old child with SM Cym033- obtained from a 23month old child with UM	Proportion of individuals with IgG specific for VSA of >1 isolate was significantly higher in the UM group compared to the SM group. Mean MFI levels of responses to the three isolates tested were significantly higher in the UM group compared to the SM group.

(Kohler et al. 2003)	Gabon	6months – 11 years	Matched case- control study (Matched on age, gender and provenance)	SM (N=100): Hb<5g/dL and/or hyperparasitemia (>250,000p/μl) with or without other signs of severe malaria UM (N=100): parasitemia (1,000-50,000p/μl), Hb>8g/dL, glycemia >50mg/dL	MSP-1 (N- terminal) MSP-1 ₁₉ MSP-2 (N- terminal) MSP-2 (Cterminal)	Proportion of individuals with IgG specific for MSP-1 (N- terminal), MSP-2(N-terminal) and MSP-2(C-terminal) was significantly higher in the SM group compared to the UM group. No difference in prevalence of IgG, IgG1 and IgG3 responses against MSP-1 ₁₉ between the two groups.
(Cissoko et al. 2006)	Mali	3months – 14 years	Matched case- control study (Matched on age, ethnicity and residence to a healthy and uncomplicated malaria control)	SM (N=197): WHO criteria for severe malaria. UM (N=206): parasites and fever or parasites and symptoms of malaria (PCD) HC (N=203)	GPI	Mean IgG and IgM levels were higher in SM or UM malaria patients than in healthy controls. Higher levels of IgG and IgM were observed in CM patients compared with HC. Elevated levels of IgM were observed in children with CM compared with UM.
(Leoratti et al. 2008)	Brazil	Median age 30.2 years	Case-control study	SM (N=70): SMA, high parasitemia, hypoglycemia (<40mg/dL) or serum creatinine levels >1.5mg/dL UM (N=148): Fever,	<i>P. falciparum</i> blood stages antigen	Levels and prevalence of IgG1, IgG2 and IgG3 were higher in the UM and AS groups compared to the SM group. IgG4 and IgE levels were higher

				parasitemia without other causes of infections or signs of severe malaria			in the SM group than in the UM and AS group.
				AS ⁷ (N=15): Parasites with no clinical symptoms.			Levels of IgM, IgG and IgA did not differ among the groups.
(Perlmann et al. 2000)	Thai-Myanmar border	16-64 years	Matched case-control study (Matched on age, sex and geographic origin)	SM (N=35): SMA or jaundice, acute renal failure, hyperparasitemia.	Schizont lysate		IgG levels were significantly higher in the UM than in the SM group.
				UM (N=35): Parasites, fever and non-severe malaria symptoms.			IgE levels were higher in the SM group but the difference was not significant.
(Schreiber et al. 2006)	Ghana	6-147 months	Matched case-control study (Matched on age, gender and village of residence)	CM (N=23): Parasitemia, BCS<3, cerebral fluid cell count <50cells/ μ L	Rifin (RIF-29) Schizont lysate		IgG and IgG subclass levels were significantly higher in the SM group than in the AS.
				SMA (N=35): Parasitemia and Hb<5g/dL			Higher IgG2 and IgG4 levels to RIF-29 in the CM cases than in the AS group. No difference in IgG2 and IgG4 levels among the SMA and AS groups.
				AS (N=58): asymptomatic infection, no history of severe malaria.			No difference in IgG1 levels to RIF-29 between the CM and SMA cases compared to the AS group.
							Significantly higher IgG3 level to RIF-29 in the CM and SMA

(Ahmed Ismail et al. 2013)	Uganda	6 months to 3 years	Case-control study	SM (N=46) According to WHO definition	AMA1 (D10) ectodomain EBA140, EBA175, EBA181 Rh2A9 Rh4A3 MSP-2 (FC27 and 3D7) MSP-1 ₁₉	IgG levels against EBA181, MSP-2 (FC27 and 3D7), AMA1 were significantly higher in UM compared to SM cases. No difference in antibody prevalence between the two groups for all antigens tested except EBA140, which was significantly higher in the SM cases compared to UM. No difference between the two groups in IgM levels against MSP-2 (FC27 and 3D7).
				UM (N=39)		

¹SM, severe malaria

²UM, uncomplicated malaria

³CM, cerebral malaria

⁴SMA, severe malaria anemia

⁵HC, healthy controls

⁶The standard WHO defined criteria for severe malaria includes severe malaria anemia, impaired consciousness or coma, prostration, multiple seizures, hyperlactatemia or metabolic acidosis, dark urine, hypoglycemia, jaundice, respiratory distress, shock, and/or renal failure (WHO, 2000)

⁷AS, asymptomatic *P. falciparum* infections

Although many studies have been conducted comparing antibody responses between severe malaria cases, uncomplicated malaria cases and healthy controls, the evidence that antibodies protect for any given antigen is inconclusive.

1.9 *Plasmodium falciparum* merozoite antigens

The studies conducted in this thesis focus on merozoite targets of protective antibodies. A panel of merozoite antigens, which are currently under development as potential vaccine candidates, were selected and are reviewed in this section. The importance of these antigens as putative targets of protective antibodies has been demonstrated in *in vitro* experiments, animal model studies, population genetic studies, prospective cohort studies and human vaccine trials. Nevertheless, the prospective cohort studies and human vaccine trials conducted thus far have focused on uncomplicated episodes of malaria and infection as endpoints of interest, therefore there is a paucity of data demonstrating the potential or lack thereof of these antigens as targets of immunity to well defined severe episodes of malaria.

1.9.1 Apical Membrane antigen 1 (AMA1)

AMA1 is an 83kDa type 1 integral protein synthesized during the late schizont stage of parasite development (Narum and Thomas 1994) and localized to the micronemes of the merozoite (Healer et al. 2002, Bannister et al. 2003). The ectodomain of the protein is made up of an N-terminal pro-sequence and three domains (DI, DII and DIII) linked together by eight disulphide bonds (Hodder et al. 1996). During schizont rupture, the prosequence is proteolytically cleaved and the mature 66 kDa form of AMA1 is translocated to the merozoite surface (Narum and Thomas 1994) where it undergoes further cleavage by the membrane-bound *P. falciparum* subtilisin-like protease 2 (*PfSUB2*) ‘sheddase’ to form the 44 and 48 kDa soluble fragments (Howell et al. 2001).

AMA1 has over 60 polymorphic sites, with the majority of them concentrated near a hydrophobic trough in domain I of the protein (Bai et al. 2005). In a study conducted in Mali, a total of 214 AMA1 haplotypes were identified among 506 predominant-clone infections

(Takala et al. 2009). Similarly, among 50 Thai (Polley, Chokejindachai and Conway 2003a) and 129 Kenyan isolates (Osier et al. 2010b) sequenced, 27 and 78 AMA1 haplotypes were identified, respectively, highlighting the highly polymorphic nature of the antigen. There is strong evidence suggesting that mutations on the AMA1 gene arise due to diversifying selection driven by immune pressure (Polley and Conway 2001, Polley et al. 2003a, Osier et al. 2010b).

AMA1 is thought to play a role in the process of RBC invasion through reorienting the merozoite after initial contact with the erythrocyte (Mitchell et al. 2004) and through initiating tight junction formation by associating with the RON proteins. In particular, the surface exposed ectodomain of RON2 binds to the conserved hydrophobic trough of AMA1 to form the moving junction (Richard et al. 2010, Alexander et al. 2006, Lamarque et al. 2011, Tonkin et al. 2011). Antibodies against AMA1 (Hodder, Crewther and Anders 2001, Healer et al. 2004, Kocken et al. 2002) or short peptides such as R1 that bind to the hydrophobic groove of AMA1 (Richard et al. 2010) block invasion into erythrocytes.

Individuals living in malaria endemic areas develop antibodies against AMA1 and these have been associated with a reduced risk of developing a clinical episode of malaria in some studies (Fowkes et al. 2010, Polley et al. 2004, Osier et al. 2008). Protective immunization in mice and monkeys has also been achieved (Collins et al. 1994, Anders et al. 1998, Stowers et al. 2002, Dutta et al. 2009) although such studies demonstrate a high degree of strain-specific protection against challenge with homologous parasite strains. Consistent with these findings, the most advanced AMA1 trial conducted in Malian children demonstrated limited overall protective efficacy against clinical episodes of malaria but conferred 64.3% protection against infection with homologous parasites (Thera et al. 2011). These observations suggest that induction of broad protection by an AMA1 vaccine requires inclusion of multiple haplotypes representative of circulating parasite strains. However, with more than 200 circulating AMA1

haplotypes (Takala et al. 2009), development of a multi-allelic AMA1 vaccine seems unachievable, although some studies have provided evidence that only a limited fraction of alleles are necessary for inclusion into a vaccine to offer broad coverage of the existing diversity and induction of strain-transcending protection (Duan et al. 2008, Drew et al. 2012, Miura et al. 2013b), or by increasing the immunogenicity of conserved epitopes within the protein (Dutta et al. 2013). These studies provide renewed hope that an effective AMA1 vaccine is attainable and the rational selection of the important haplotypes for inclusion will inform design of future AMA1 vaccines.

1.9.2 Merozoite surface protein -2 (MSP-2)

MSP-2 is a 45 to 52 kDa protein anchored on the surface of the merozoite by a glycosylphosphatidylinositol (GPI) anchor. MSP-2 consists of a central variable region, which is flanked by highly conserved N and C terminal regions (Smythe et al. 1991, Fenton et al. 1991). The central repeat region is highly polymorphic, varying in length, number and sequence among isolates, flanked by non-repetitive dimorphic sequences that define two major allelic families – 3D7-like and FC27-like (Smythe et al. 1991). The 3D7-like family contains 4-8 amino acid repeats with five different amino acids being used whereas the FC27-like family has 12 and 32 amino acids repeated up to 4 times (Felger et al. 2003).

The observation that this gene is highly polymorphic suggests that this is driven by immune pressure, a hypothesis that has been confirmed by some population genetic studies (Barry et al. 2009, Conway 1997). For instance, in the study by Conway *et al.*, MSP-2 alleles from three geographical locations were tested using the Ewens-Wattersons test of neutrality and the F statistic from this test was lower than that obtained under neutrality, demonstrating evidence of positive selection on this gene (Conway 1997). In early studies, monoclonal antibodies

raised against asexual blood-stage parasites were able to recognize MSP-2 and inhibit parasite growth *in vitro* suggesting that this protein plays a role during the process of invasion (Epping et al. 1988). Such studies paved the way for further characterization of the protein as a potential vaccine candidate. Mice immunized with the N- and C-terminal regions of MSP-2 were partially protected against lethal *P. chabaudi* challenge (Saul et al. 1992).

Naturally-acquired antibodies to MSP-2 are present in individuals living in malaria endemic areas (Taylor et al. 1995, Polley et al. 2006) with a predominance of the IgG3 subclass (Taylor et al. 1995, Metzger et al. 2003, Tongren et al. 2005) and mainly directed to the central variable repeat region (Taylor et al. 1995). These antibodies are associated with protection against clinical episodes of malaria or infection in some immuno-epidemiological studies (Polley et al. 2006, Osier et al. 2008, Taylor et al. 1998) and not others (Scopel et al. 2007) but no clear evidence of allele-specific protection has been observed (Osier et al. 2010a, Stanisic et al. 2009). Further testing of MSP-2 as a component of a combination vaccine in a Phase 1/2 trial in Papua New Guinea, revealed protection against infection with parasite strains contained in the vaccine (Genton et al. 2002). This observed strain-specific protection led to testing of a vaccine containing both MSP-2 alleles in healthy malaria naïve adults resident in Australia, a trial that was halted due to reactogenicity to the vaccine formulation (McCarthy et al. 2011). However, analyses of serum samples collected from vaccinees in this trial showed induction of high antibody levels to both forms of MSP-2 and these antibodies had potent ADCI but not GIA activity (McCarthy et al. 2011), suggesting that MSP-2 is a potential target of functional antibodies that could induce protection.

1.9.3 Merozoite Surface Protein 3 (MSP-3)

MSP-3 is a soluble polymorphic antigen associated with the merozoite surface. At the sequence level, it has twelve copies of heptad repeats (AXXAXXX) in three blocks in the N-terminus forming an alpha helical coil/coil motif and a leucine zipper sequence at the C-terminus (McColl et al. 1994). Polymorphisms within the N-terminal domain arise due to insertions and deletions in non-repetitive sequences within and flanking the alanine heptad-repeat domain (McColl et al. 1994) and the polymorphisms are mainly driven by selection pressure (Polley et al. 2007). MSP-3 sequences can be classified into two major allelic classes, the 3D7 and K1 types (Huber et al. 1997).

Manipulation of the MSP-3 gene leading to loss of the leucine zipper motif interfered with trafficking of MSP-3 and a related protein (acidic-basic repeat antigen) to the merozoite surface resulting in reduced invasion into erythrocytes (Mills et al. 2002), findings that imply that MSP-3 is not very essential during invasion and intra-erythrocytic development. However, antibodies from malaria immune adults directed to MSP-3 inhibited parasite growth *in vitro* in the presence of monocytes (Oeuvray et al. 1994) and field studies have also demonstrated that anti-MSP-3 antibodies are associated with a reduced risk of developing malaria (Polley et al. 2007, Osier et al. 2007, Soe et al. 2004, Meraldi et al. 2004). Similarly, studies done in *saimiri* and *aotus* monkeys have demonstrated protection against lethal *P. falciparum* challenge in animals immunized with MSP-3. These observations provide evidence that MSP-3 is a target of protective antibodies. Currently, MSP-3 trials in humans are underway, testing the C-terminal domain in combination with GLURP antigen and results from Phase 1/2b trials demonstrate a good safety profile and induction of high levels of functional antibodies in adults and children living in a malaria endemic area (Mordmuller et al. 2010, Jepsen et al. 2013, Belard et al. 2011).

1.9.4 Merozoite surface protein 1 (MSP-1)

MSP-1 is the most abundant protein on the surface of the merozoite. It is synthesized during the schizont stage of development as a 190 kDa precursor protein and undergoes proteolytic cleavage by *P. falciparum* subtilisin 1 (*PfSUB1*) into four polypeptides (p83, p30, p38 and p42), which remain non-covalently linked and anchored to the merozoite surface by GPI residues (McBride and Heidrich 1987, Holder et al. 1992). At the time of invasion, the 42 kDa protein undergoes secondary processing into 33 and 19 kDa fragments by the *P. falciparum* subtilisin 2 (*PfSUB2*) enzyme (Blackman and Holder 1992, Blackman et al. 1990). MSP-1₃₃ is shed from the surface together with associated proteins and the other MSP-1 polypeptides, whereas MSP-1₁₉ remains membrane bound and is carried into erythrocytes during invasion (Blackman, Whittle and Holder 1991).

MSP-1 consists of seventeen distinct blocks that are either variable, conserved or semi-conserved (Tanabe et al. 1987). The N-terminal block 2 region displays the greatest sequence variability (Miller et al. 1993) with the different variants at this locus grouped into 3 classes (K1, MAD20 and RO33) based on the parasite strain from which the sequences were first identified. For the purpose of this thesis, antibody responses to the conserved 19 Kda C-terminal fragment were measured, but there is evidence that other regions of MSP-1 can elicit protective immune responses. For instance, naturally-acquired antibodies to the N-terminal block 2 are associated with a reduced risk of developing malaria in some studies (Conway et al. 2000, Polley et al. 2003b) but not others (Gray et al. 2007, Osier et al. 2008), and in a primate model of malaria, immunization with the N-terminal domain elicited partial protection against lethal challenge with the FVO parasite strain (Cavanagh et al. 2014).

Polyclonal (Chang et al. 1992) and monoclonal antibodies (Blackman et al. 1990, Chappel and Holder 1993) and sera from immune adults (Egan et al. 1999, O'Donnell et al. 2001) targeting MSP-1₁₉ can inhibit parasite growth *in vitro* by blocking secondary processing of MSP-1₄₂

(Blackman et al. 1994). Of note also is that antibodies to different epitopes of MSP-1₁₉ can either be inhibitory, blocking or neutral, with blocking antibodies interfering with the binding of inhibitory ones hence allowing secondary processing of the protein (Blackman et al. 1994, Uthaipibull et al. 2001). If MSP-1₁₉ is the specific target of antibodies that inhibit secondary processing of the protein, what is the contribution of responses to the MSP-1₄₂ fragment as a whole? Do these responses exhibit superior protection than those directed at MSP-1₁₉? There is some evidence that MSP-1₁₉ has limited T-cell epitopes (Egan et al. 1997) whereas the region upstream of MSP-1₁₉ that is present in MSP-1₄₂ contains dominant T-cell epitopes (Udhayakumar et al. 1995) suggesting that MSP-1₄₂ could be a better immunogen. Studies to address this question have yielded conflicting findings, for instance, in a primate model of malaria, monkeys immunized with MSP-1₄₂ were protected from lethal challenge with malaria (Stowers et al. 2001, Chang et al. 1996) but immunization with MSP-1₁₉ in the same study (Stowers et al. 2001) and others (Burghaus et al. 1996, Kumar et al. 2000) was not protective due to induction of very low antibody titres. The opposite effect has been reported in other studies, where immunization with MSP-1₁₉ yielded better protection than immunization with MSP-1₄₂ (Kumar et al. 1995). Thus far, it is not clear whether MSP-1₄₂ is more protective than MSP-1₁₉.

Surprisingly, out of nine immuno-epidemiological studies assessing the protective efficacy of antibodies to the C-terminus of MSP-1 in malaria exposed populations (Stanisic et al. 2009, Egan et al. 1996, Osier et al. 2008, Conway et al. 2000, Cavanagh et al. 2004, al-Yaman et al. 1996, Nebie et al. 2008a, Dodo et al. 2008, Perraut et al. 2005b) only one study specifically looked at naturally-acquired antibodies to MSP-1₄₂ and demonstrated a protective effect of these antibodies (al-Yaman et al. 1996). All other studies have examined responses to MSP-1₁₉ albeit yielding conflicting findings. In half of the studies, antibodies to MSP-1₁₉ were associated with a significant reduction in risk of developing malaria (Perraut et al. 2005b,

Egan et al. 1996, Doodoo et al. 2008, Stanisic et al. 2009) and in the other half; MSP-1₁₉ antibodies were not protective (Osier et al. 2008, Conway et al. 2000, Cavanagh et al. 2004, Nebie et al. 2008a). In a recent meta-analysis of merozoite targets of protective immunity, MSP-1₁₉ was significantly associated with an overall protective efficacy of 18% from pooled analyses (Fowkes et al. 2010). In view of the fact that immuno-epidemiological studies are important tools used to inform the candidate antigens to put forward for vaccine development, studies examining responses to MSP-1₁₉ seem to outcompete those testing MSP-1₄₂ but surprisingly, the latter has preferentially been taken forward for testing in human trials. To the best of my knowledge, there's a paucity of field studies to support the importance of MSP-1₄₂ as a target of protective immunity and it is not surprising therefore that although the vaccine was safe and immunogenic when tested in adults in Kenya (Withers et al. 2006), United States (Ockenhouse et al. 2006) and Mali (Thera et al. 2006), it did not elicit protection in a paediatric trial in Kenya (Ogutu et al. 2009). It is thought that vaccine failure was due to lack of strain-transcending protection given that only one allele (3D7) of MSP-1₄₂ was included in the vaccine. MSP-1₁₉ has been tested together with AMA1 but although the vaccine was safe and immunogenic (Hu et al. 2008), the growth inhibition activity of these antibodies was suboptimal (Malkin et al. 2008) and the current status of trials of this chimeric protein is inactive (Schwartz et al. 2012).

1.9.5 *Plasmodium falciparum* reticulocyte binding-like homologue protein 2 (*PfRh2*)

PfRh2 is a hydrophilic protein expressed during the merozoite stage of parasite development and localized to the rhoptries. It shares 500 amino acid sequence homology with its *P. vivax* homologue, *PvRh2* (Rayner et al. 2000). The *PfRh2a* and *PfRh2b* genes are identical at the 5' end for approximately 8kb of their sequences but are highly divergent at the 3' end, presumed

to have arisen due to evolutionary duplication. The unique sequences span part of the ectodomain, a putative transmembrane domain and the cytoplasmic tail region (Rayner et al. 2000, Triglia et al. 2001). The N-terminal region of the *PfRh2* gene is highly polymorphic (Rayner et al. 2005, Reiling et al. 2010) and some evidence suggests that this region is under diversifying selective pressure (Reiling et al. 2010).

In an earlier study, the erythrocyte binding capacity of *PfRh2* could not be detected (Triglia et al. 2001) but more recently, there's evidence that *PfRh2* binds erythrocytes via a sialic acid-independent, chymotrypsin-sensitive and trypsin-resistant pathway (Sahar et al. 2011, Duraisingh et al. 2003). Using gene knockout experiments, Duraisingh *et al.* demonstrated that *PfRh2b* but not *PfRh2a* mediates invasion of erythrocytes (Duraisingh et al. 2003) and blocking this ligand impairs invasion capacity. These observations, together with limited data from epidemiological studies showing that *PfRh2* antibodies are significantly associated with protection against high density parasitemia and clinical episodes of malaria (Reiling et al. 2010), suggest that it could be an important target of protective immunity. For the purpose of this thesis, I examined antibody responses to Rh2A9, a fragment of *PfRh2* spanning the 2030 – 2528 amino acid positions, which was demonstrated to induce high antibody titres and had the highest seroprevalence among Papua New Guinean children (Reiling et al. 2010).

1.10 Aims and scope of the thesis

Naturally-acquired malaria immunity has many determinants and in the absence of well-defined targets and correlates of protection against *P. falciparum*, the studies presented in this thesis have focused separately on the clinical endpoints of uncomplicated and severe malaria incidence as an approach towards defining acquisition of immunity. The overall aim is to identify the potential merozoite targets of antibodies and the protective mechanisms associated with NAI.

Below, I outline the aims, rationale and a brief summary of the findings presented in the results chapters of this thesis.

In **Chapter Three**, the initial aim was to compare two widely used ELISA methods of quantifying antibody responses to blood-stage antigens. I demonstrate the limited utility of measuring and reporting the magnitude of antibody responses as ELISA OD values particularly in settings of high malaria transmission intensity, and outline a quantification method using a standard reference reagent to standardize measurement of antibody titres.

In **Chapter Four**, I set out to define threshold antibody concentrations against specific merozoite antigens that are associated with a reduced risk of developing a clinical episode of malaria and their application to prospective cohort studies. Although there is extensive literature supporting several merozoite antigens as targets of immunity, for any given antigen, there has been conflicting data with some studies demonstrating protection while others do not (Fowkes et al. 2010). The numerous potential reasons for these discrepancies have been elaborated in this thesis but the finding in our own setting that antibodies to merozoite antigens were associated with protection in one cohort but not in another, provided the rationale to explore possible explanations for this paradox. Using the ELISA quantification method outlined in chapter three, antibodies to similar antigens were quantified in a high transmission cohort where antibodies were shown to be protective and a low transmission cohort where similar antibodies were not associated with protection, all based on a cutoff of responses above the reactivity of malaria naïve sera (seropositivity). Using a statistical model, threshold concentrations associated with a reduced risk of developing a clinical episode of malaria to various antigens were defined in the high transmission cohort and applied to the low transmission cohort. Children who achieved these thresholds had a lower risk of developing malaria, providing evidence that differences in transmission intensities notwithstanding, achieving particular thresholds are more important for protection and may be useful correlates

of protection for future studies. I extended this work in post-hoc analyses and outline an interesting finding of similar threshold concentration cutoffs derived independently in three cohorts (Chonyi, Junju and Tanzania). This observation suggests that achieving the proposed threshold levels may be clinically and/or biological relevant and could form useful correlates of protection, given the demonstration that achieving these titres was associated with a reduced risk of developing uncomplicated malaria in two geographically distinct populations.

Chapter Five focuses on immunity to severe malaria. Using a case-control approach nested within a longitudinally monitored birth cohort of children followed up every three months from birth upto two years of age for development of severe malaria, I have i) described the dynamics of antibodies to a panel of merozoite antigens and ii) determined the association between specific antibodies with protection against severe malaria during the first two years of life.

First, as outlined in section 1.5.2 above, the paucity of epidemiological data on the role of antibodies in protection against well-characterized severe episodes and more importantly, the protective targets from the numerous *Pf* proteins provided the rationale for this study. Second, there have been few longitudinal studies investigating the acquisition of antibodies to malarial antigens during childhood using repeated antibody measurements over time. Antibodies are relatively short-lived in children and the measurement of antibody responses at a single time point may therefore inaccurately reflect the ability of an individual to mount an immune response. The longitudinal sampling of this cohort of children provided the unique opportunity to evaluate, to my knowledge, the first description of the association between an individual's overall response in protection against severe malaria. There was no evidence of an association between antibodies against the five merozoite antigens tested in reducing the risk of developing severe malaria during the first two years of life with the exception of AMA1.

Antibodies to AMA1 alleles were associated with a significant reduction in risk of disease using samples collected immediately prior to the disease episode and a general trend towards reduced risk when the mean response of all samples collected from an individual were analysed. I also demonstrate the prevalence of low and stable antibody titres over time in this group of children, boosted in the presence of concurrent parasitemia. This finding may be explained by inadequate exposure following the decline in malaria transmission intensity during the period of the study and possibly due to intrinsic factors associated with low host age.

Chapter Six focuses on passively transferred maternal antibodies with the specific aim of describing the decay of maternal antibodies against a panel of merozoite antigens and determining the role of these antibodies in protection against severe malaria and infection during the first six months of life. The rarity of clinical episodes of malaria in infants during the first few months of life is thought to be mediated in part by the presence of antibodies transferred to the infant from the mother. However, the evidence for a role of these antibodies in protection has been inconsistent between studies. In addition, no study so far, to the best of my knowledge has specifically evaluated protection against severe episodes of malaria. The key findings from this chapter are i) transplacentally acquired anti-merozoite antibodies have a half-life of between 1 month and 34 months, varying depending on the antigen tested; ii) the rate of decay of maternal antibodies differs according to the level of antibodies - high titre antibodies were shown to decay relatively faster than low titre antibodies; and iii) maternal antibodies were markers of exposure to infection and not associated with protection against asymptomatic infection or severe malaria.

Chapter Seven examines the role of antibody-mediated functional activity in protection against severe malaria using the GIA and ADRB assays. The lack of *in vitro* measures that

correlate with *in vivo* protection against malaria have partly hampered the identification of potential targets associated with naturally-acquired immunity. This, together with the lack of prospective studies examining the association between the functional activity of antibodies and protection against severe malaria, provided the rationale for conducting this study. I have described the optimization of a recently described ADRB assay that was shown to correlate with immunity against clinical malaria in Senegal and also showed an interesting and novel finding that children who had a combination of antibodies mediating both GIA and ADRB had significantly reduced odds of developing severe malaria. Previously, most studies have only concentrated on one functional mechanism. These findings therefore highlight the need to assess multiple mechanisms in concert and provide a useful platform to evaluate their antigenic targets as potential vaccine candidates.

Finally, in **Chapter eight** I provide a summary of the findings generated in this thesis and recommendations for future studies.

CHAPTER TWO

Materials and Methods

2.0 Study area

The study was conducted in Kilifi County, situated 60 km north of Mombasa city, along the Kenyan coast. According to the Kenya population and housing census report released in August 2010, the county has a population of 1,109,735 residents and covers an area of 12,609 km² (<http://www.knbs.or.ke/counties.php>). The majority of the inhabitants belong to the Giriama subgroup, one of the nine subgroups of the Mijikenda ethnic group. The area experiences two annual rainy seasons, April-July and November-December during which most of the malaria transmission occurs. The main vectors that transmit malaria are *Anopheles gambiae s.l.* and *Anopheles funestus* species (Mbogo et al. 2003).

2.1 Study population

Participants in this study were children drawn from the Kilifi Health and Demographic Surveillance System (KHDSS) which was established in the year 2000 and covers approximately 900km² around Kilifi District Hospital (KDH). The area covered by the KHDSS consists of 15 administrative locations, 40 sub locations, 186 enumeration zones and 34,795 households (Figure 2.1) (Scott et al. 2012). The KHDSS was established to provide a link between data collected from the hospital and the demographic characteristics of residents in order to i) provide a sampling framework for epidemiological studies; ii) monitor deaths, births and migration patterns; iii) describe the burden of local childhood illness; and iv) evaluate the impact of interventions against the most prevalent childhood infections. The KHDSS tracks a population of 260,000 residents who are monitored quarterly for births,

2.2 Study design

2.2.1 Kilifi Birth Cohort

The study was a matched case-control nested within a longitudinally monitored birth cohort (Kilifi Birth Cohort) set up in 2001 to establish the risk factors for invasive pneumococcal disease in young children. Ethical approval was granted by the Kenya National Ethics Review Committee (SCC 613 - A cohort study of susceptibility to invasive pneumococcal disease among children aged 0-23 months). Infants born to mothers resident within the KHDSS were recruited at birth (at the maternity clinic) or within their first month of life during their visit to the vaccination clinic. At recruitment, 2ml of cord blood was collected and the child was given a unique identifying number (Kilifi Birth Cohort number), which was used to identify the child during subsequent visits. No blood was collected from infants recruited during their visit to the vaccination clinic. After every 3 months, the children were seen at the outpatient clinic where 1.5ml of saliva and 2ml of venous blood was collected regardless of whether the child was ill or well. These follow-up visits were concluded when the child reached 2 years of age. Children who did not attend a scheduled 3-monthly visit were followed up at home by a fieldworker the following day and re-invited to attend the clinic. Those who were unavailable during the home visit and did not attend the subsequent appointment were sought at home and similarly re-invited to attend the clinic. If they did not attend two consecutive 3-monthly visits, they were withdrawn from the study and replaced with a new recruit.

Children from within the cohort who developed an illness requiring hospital admission were identified using the unique Kilifi Birth Cohort number assigned to them during recruitment. Detailed clinical and laboratory investigations were carried out to ascertain the cause of illness

using well-defined case definitions as per the national and WHO clinical guidelines for management and referral of common conditions at level 4 – 6 hospitals (WHO 2009).

2.2.2 Matched case-control study

A matched case-control study was nested within the Kilifi birth cohort. Children from the cohort who were admitted to the high dependency unit between April 2002 and January 2010 with well-defined severe malaria were selected as cases. Severe malaria was defined as admission to hospital with detectable parasites by microscopy and either one of the following symptoms: i) hemoglobin $<5\text{g/dL}$; ii) Blantyre coma score <5 ; or iii) deep breathing or chest indrawing (Marsh et al. 1995). Children fulfilling the criteria for severe malaria but in addition having positive cultures from blood or cerebrospinal fluid and/or a white blood cell count $>10/\mu\text{l}$, were excluded from the study to avoid misclassification with other potential causes of severe illness in which case the parasitemia may have been coincidental.

Cases were individually matched to at least three controls by age (± 4 months); residence and cohort visit dates (± 4 months) to minimize potential confounding due to differences in cumulative exposure to infection which influences the levels of antibodies and also increases the risk of developing disease. At least three controls per case were selected to increase the statistical power of the study (Miettinen 1969). Controls were selected from cohort participants who were never admitted to hospital with severe malaria up to the time of admission of the index case.

2.2.3 Adult serum samples

For the well-studied merozoite antigens, adults living in malaria endemic areas have high antibody prevalence and a large proportion mount high titre responses compared to young children (Polley et al. 2004, Taylor et al. 1998, Ramasamy, Nagendran and Ramasamy 1994, McCallum et al. 2008). Adults were therefore recruited to allow for comparison of antibody levels and function with infants recruited in the case-control study.

Two cross-sectional surveys were conducted in December 2007 and August 2008 in which 26 and 75 individuals were recruited; respectively. The adults were aged between 18 – 65 years and residing within Junju sub-location, Kilifi County. Following informed consent, individuals were requested to attend the outpatient department of Kilifi District Hospital for a physical examination performed by the study clinician and to donate 15ml of venous blood drawn into 15ml falcon tubes containing heparin. A small volume of blood (1ml) was also transferred into EDTA tubes for complete blood counts. Thick and thin blood smears were also performed to detect *P. falciparum* infections. The blood samples were processed immediately after collection. Serum and peripheral blood mononuclear cells (PBMCs) were separated and either used fresh in assays or stored at -80°C.

2.3 Laboratory methods

2.3.1 Recombinant *P. falciparum* antigens

The recombinant antigens used in the study were based on allelic sequences of various *P. falciparum* parasite strains and were expressed in *Escherichia coli* or *Pichia pastoris*. MSP-2 antigens were based on the Dd2 and CH150/9 parasite strains and were expressed as glutathione *S*-transferase (GST) fusion proteins in *E. coli*. The 19-KDa fragment of MSP-1₁₉

was expressed as a GST-fusion protein and was based on the Wellcome parasite strain. Transformed *E. coli* cells containing the MSP-2 and MSP-1₁₉ genes were generously provided by Dr. Kevin Tetteh (London School of Hygiene and Tropical Medicine). Full-length MSP-3 antigens were expressed as maltose binding protein (MBP) – fusion proteins in *E. coli* and were based on the 3D7 and K1 parasite strains. Full-length recombinant AMA1 ectodomains based on the FVO and 3D7 parasite strains were generously provided by Dr. Edmond Remarque (Biomedical Primate Research Centre, Netherlands). The AMA1 antigens were expressed in *Pichia pastoris* as His-tagged proteins. A fragment of *PfRh2* based on the 3D7 parasite strain was expressed in *E. coli* as a GST-fused protein (Reiling et al. 2010). Transformed *E. coli* cells containing the *PfRh2* fragment were generously provided by collaborators in Prof. James Beeson's laboratory (Burnet Institute of Medical Research, Australia). A *P. falciparum* schizont lysate based on the A4 parasite line was also tested. The lysate was prepared by sonicating a highly synchronous culture containing mature schizonts at high speed. The lysate was stored frozen at -80°C.

Table 2.1: Details of the recombinant *P. falciparum* antigens used in the study

Antigen	Sequence Region	Tag	Expression System	Reference
MSP-2 (Dd2)	22-247	GST	<i>E. coli</i>	(Taylor et al. 1995)
MSP-2 (CH150/9)	1-184	GST	<i>E. coli</i>	(Taylor et al. 1995)
MSP-3 (3D7)	2-354	MBP	<i>E. coli</i>	(Polley et al. 2007)
MSP-3 (K1)	2-379	MBP	<i>E. coli</i>	(Polley et al. 2007)
AMA1 (3D7)	83-531	His	<i>P. pastoris</i>	(Dutta et al. 2002)
AMA1 (FVO)	25-544	His	<i>P. pastoris</i>	(Kocken et al. 2002)

<i>PfRh2</i>	2030-2528	GST	<i>E. coli</i>	(Reiling et al. 2010)
MSP-1 ₁₉	1631-1726	GST	<i>E. coli</i>	(Burghaus and Holder 1994)

2.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

Two different ELISA techniques were used to measure antibody titres in serum samples and are described below:

2.3.2.1 Standard ELISA

A well-established standard ELISA protocol was used to measure antibody titres against the recombinant antigens (Polley et al. 2004, Polley et al. 2006, Osier et al. 2008) and *P. falciparum* schizont extract (Ndungu et al. 2002). Wells of Dynex 4HBX Immunolon plates (Dynex technologies Inc) were coated with 100µl of 0.5µg/ml recombinant antigen diluted in coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.4-9.6) or 100µl of schizont extract diluted 1:1000 as previously described (Ndungu et al. 2002). After an overnight incubation at 4°C, plates were washed four times in 1X PBS containing 0.05% Tween-20 (wash buffer) and blocked for 5 hours at room temperature with 1% skimmed milk diluted in PBS Tween-20 (blocking buffer). The plates were washed four times in wash buffer and 100µl of serum diluted 1:1000 in blocking buffer added to each well. Following an overnight incubation at 4°C, wells were washed and incubated for 3 hours at room temperature with 100µl of HRP conjugated polyclonal rabbit anti-human IgG (Dako) diluted 1:5000 in blocking buffer. The wells were washed four times and incubated at room temperature with 100µl of development buffer (0.1M citric acid, 0.2M Na₂HPO₄, 4mg *o*-Phenylenediamine dihydrochloride tablets

(Sigma), 8µl hydrogen peroxide and 5ml distilled water). After 25mins, the reaction was stopped with 25µl H₂SO₄ and absorbance read at 492nm. Eleven serial dilutions of a purified immunoglobulin reagent prepared from a pool of Malawian adults presumed to be malaria-immune (Taylor et al. 1992) were included for every antigen tested to obtain a standard dilution curve that allows conversion of optical density readings to relative antibody concentrations. A pool of sera obtained from Kilifi adults was included on a single well in each plate as a positive control to allow for standardization of day-to-day and plate-to-plate variation. Twenty sera obtained from malaria non-exposed UK adults were also included as negative controls for each antigen tested. All samples were assayed in duplicate and those that had a coefficient of variation (CV) greater than 20% were repeated.

2.3.2.2 Multiplex bead-based antibody assay

A multiplex bead-based ELISA assay was used to simultaneously quantify antibodies against multiple *P. falciparum* merozoite antigens (Fouda et al. 2006). Antigens were coupled onto different MagPlex® microspheres (Luminex corporation) each bearing a unique spectral address. The coupling reaction was performed using the Bio-Plex amine coupling kit (BioRad). An aliquot of one million beads was washed in 100µl wash buffer by vortexing for 30 seconds and resuspended in 80µl of bead activation buffer. 10µl of a 50mg/ml solution of sulfo-N-hydroxysulfosuccinimide (S-NHS) and 10µl of a 50mg/ml solution of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDAC) diluted in bead activation buffer were each added to the beads and incubated at room temperature in the dark for 20min on a rotator. The activated beads were washed and resuspended in 100µl of 1X PBS pH 7.4 followed by addition of 1µg of recombinant antigen per million beads. The beads were incubated for 2 hours at room temperature on a rotator, washed with 1X PBS pH 7.4 and

blocked with 250µl of blocking buffer for 30min at room temperature. The coupled beads were washed once in storage buffer, resuspended in 150µl of the same buffer and stored 4°C.

Microspheres were resuspended by vortexing and diluted to 100 beads/µl using PBS with 1% bovine serum albumin (BSA) (dilution buffer). 50µl of diluted beads and 50µl of plasma (diluted 1:1000 in dilution buffer) were added to individual wells of Bio-Plex pro flat bottom plates (BioRad). The plates were incubated on a microplate shaker (rotation 500 rpm) for 1 hour at room temperature. The plates were washed five times with 1X PBS containing 0.05% Tween-20 (wash buffer) and resuspended in 100µl of R-phycoerythrin-conjugated, f(ab')₂ fragment-specific, goat anti-human immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) diluted 1:300 in dilution buffer. After 30 min incubation at room temperature on a microplate shaker, the plates were washed five times with wash buffer. The beads were resuspended in 100µl of dilution buffer and analyzed on a Magpix® (Luminex corporation) platform using the xPONENT® software. The reader was programmed to measure fluorescence intensity of 100 beads per spectral address and the readings are expressed as median fluorescent intensity (MFI). Eleven serial dilutions of the purified immunoglobulin reagent mentioned in section 2.3.2.1 above (Taylor et al. 1992) were included for every antigen tested to obtain a standard dilution curve that allowed the conversion of MFI readings to relative antibody concentrations. A pool of sera obtained from Kilifi adults was included on a single well in each plate as a positive control to allow for standardization of day-to-day and plate-to-plate variation. Twenty sera obtained from malaria non-exposed UK adults were also included as negative controls for each antigen tested. All samples were assayed in duplicate and those that had a coefficient of variation (CV) greater than 20% were repeated.

2.3.3 *In vitro* *P. falciparum* parasite culture

Parasite strains were frozen in glycerolyte and stored in liquid nitrogen at the Jenner Institute, University of Oxford and KEMRI, Kilifi laboratories. To establish parasite cultures for antibody-mediated functional assays, parasites were retrieved from liquid nitrogen, allowed to thaw at room temperature and transferred to 50ml falcon tubes. Two hundred microlitres of 12% sodium chloride solution was added dropwise while gently shaking the tube. After 5 minutes incubation at room temperature, 10ml of 1.8% sodium chloride solution was added dropwise while gently shaking the tube, followed by incubation for 5 mins at room temperature. A final 10ml volume of 0.9% sodium chloride solution was added to the tube and the cells pelleted by centrifugation at 830 x g for 5 mins. The supernatant was discarded and the cell pellet washed twice with RPMI-1640 medium (pH 7.2) supplemented with 1mM L-glutamine, 37.5mM HEPES, 200mg/ml glucose, 100µM hypoxanthine and 25µg/ml gentamicin (incomplete media). After the final wash, 10ml of incomplete media supplemented with 10% pooled human serum was added and the parasites transferred to a sterile culture flask. A gas mixture containing 92% N₂, 5% O₂ and 3% CO₂ was also added to the parasites and the flask transferred to an incubator maintained at 37°C. Parasite cultures were routinely maintained at 1-2 % parasitemia and 2% hematocrit by adding fresh O+ erythrocytes from healthy malaria non-exposed donors.

To obtain highly synchronized parasites, culture pellets were either treated with warm 5% D-sorbitol (Sigma) solution for 10 mins at 37°C to obtain ring stage parasites or resuspended in incomplete media and passed through a magnetic column (varioMACS™) to allow separation of trophozoite stage parasites. Alternatively, a 1ml culture pellet was overlaid on a gradient containing 65% percoll (diluted in PBS) and centrifuged at 1,870 x g for 5 mins at room temperature. The brown layer containing trophozoites and schizonts between the culture

medium and the percoll solution was collected and washed twice in incomplete media at 830 x g for 5 mins at room temperature.

2.3.4 Isolation of parasitophorous enclosed merozoite structures from *P. falciparum* cultures

Parasites were maintained at 10 – 15% parasitemia and 2% hematocrit and synchronized by sorbitol lysis, as described in section 2.3.3 above, for two consecutive cycles. Synchronous ring-stage parasites were allowed to mature to trophozoite stage and enriched on a 65% percoll gradient, as described in section 2.3.3 above. The parasites were pelleted and washed twice with incomplete media at 830 x g for 5 mins. The cell pellet was resuspended in 10ml of complete media containing 10 μ M trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) (Sigma), transferred to a sterile culture flask and incubated for six to eight hours at 37°C to obtain schizont stage parasites. Schizonts were pelleted by centrifugation at 2000 rpm for 5 mins and resuspended in 1X PBS. Ten microlitres of resuspended parasites was transferred to a FastRead™ hemocytometer slide (Immune Systems Ltd) and the number of schizonts estimated as follows (<http://fastread.co.uk>): -

$$\text{No. of schizont/ml} = \text{No. of schizonts in 16 squares} \times 10^4$$

The resuspended parasites were aliquoted into eppendorf tubes, each containing approximately 1ml of 18.5×10^5 schizonts/ml and frozen at -20°C.

2.3.5 Antibody function assays

Two different assays were performed to measure the functional activity of human antibodies using the *P. falciparum* 3D7 laboratory isolate. The one-cycle growth inhibition and antibody dependent respiratory burst (ADRB) assays were performed at the Jenner Institute, whereas the two-cycle growth inhibition assay was performed in Kilifi at the KEMRI Wellcome Trust laboratories.

2.3.5.1 Growth Inhibition Assays

The ability of human serum to inhibit *in vitro* growth of parasites was measured after one or two cycles of parasite replication according to previously established protocols (Persson et al. 2006, Malkin et al. 2005). Fifty microlitres of sera were dialyzed to remove antimalarial drugs prior to assay set-up using 20KDa MWCO slide-A-lyzer mini dialysis tubes (Thermo fisher scientific). Sera were dialyzed against two changes of 1X PBS each lasting 1 hour and a final overnight dialysis at 4°C. After dialysis, the samples were transferred to Amicon Ultra - 0.5 centrifugal filter devices, 100,000 MWCO (Millipore) and reconstituted back to the original volume by centrifuging at 10,000rpm for 20mins. Thereafter, sera were heat-inactivated by incubating in a water bath for 30mins at 56°C.

In the two-cycle GIA, highly synchronous trophozoite parasites were obtained (as described above) and adjusted to 0.3- 0.5% parasitemia by addition of fresh O+ erythrocytes. Parasites were resuspended at 1% hematocrit in incomplete media supplemented with 10% pooled human serum. 45µl of parasite suspension was added to individual wells of sterile 96-well U-bottom plates (Falcon) excluding the outer wells of the plate, which were filled with sterile water for humidification purposes. Thereafter, 5µl of dialyzed test sera were added into the

wells in duplicate and the plates placed in a well-sealed humidified gas chamber and incubated at 37°C. Duplicate wells with a pool of sera from non-immune UK adults were included as a negative control and 10mg/ml of a pool of purified IgG obtained from healthy Malawian adult donors (MIG) as a positive control. Several wells containing untreated cultures were also included on every plate to monitor the growth of parasites. After 40 hours incubation, 10µl of fresh complete media was added to the wells and the plates incubated for a further 40 hours. At the end of the second growth-cycle, 100µl of 10µg/ml ethidium bromide (molecular grade) diluted in 1X PBS was added to each well and the plates incubated for 30 mins at room temperature in the dark. The plates were centrifuged at 1200rpm for 1 min and the pellet resuspended in 200µl of 1X PBS. Parasitemia was determined by analyzing 70,000 events acquired on an FC500 (Beckman Coulter) flowcytometer.

Results were analyzed using FlowJo (Treestar) software and expressed as follows (Rono et al. 2012): -

$$\% \text{ GIA} = 100\% - \left\{ \frac{\text{Average parasitemia of test sample}}{\text{Average parasitemia of negative control}} \times 100 \right\}$$

In the one-cycle GIA, trophozoite stage parasites were obtained as described above and adjusted to 0.5% parasitemia by adding fresh O+ erythrocytes. The cells were pelleted and resuspended at 1% hematocrit in complete media. 36µl of parasite suspension was added to individual wells of sterile half area 96-well flat-bottom plates (Corning). Thereafter 4µl of dialyzed and heat-inactivated test sera were added to the wells in triplicate and incubated in an improvised humidified gas chamber at 37°C. In every plate, triplicate wells containing a pool of sera from non-immune UK adults were included as a negative control and 10mg/ml of purified MIG as a positive control. Wells containing uninfected O+ erythrocytes in complete

media were also included on every plate. The outer wells and inter-well spaces were filled with sterile water to minimize evaporation. After 40 hours incubation, the wells were washed once with 100µl of ice-cold 1X PBS. Parasitemia was determined by a biochemical assay that measures the amount of parasite lactate dehydrogenase (pLDH) present in mature stage parasites. Fifty millilitre stocks of LDH substrate buffer were prepared by mixing 5ml 1M Tris HCl (pH 8.0), 45ml dH₂O, 0.28g sodium L-lactate (Sigma) and 0.125ml Triton X-100 (Sigma). The stocks were stored at -20°C. On the day prior to usage, a 10mg/ml Nitro Blue Tetrazolium (NBT) tablet (Sigma) was dissolved in 50ml of LDH buffer. The substrate was prepared by mixing 10ml of LDH substrate buffer, 200µl of 50units/ml diaphorase solution (Sigma), 50µl of 10mg/ml 3-Acetylpyridine Adenine Dinucleotide (APAD) solution (Sigma), and added to individual wells (100µl each). The plates were incubated on a Titramax 100™ flat bed shaker (Heidolph) for 15 minutes. Optical density readings were measured at 650nm on a Varioskan flash multimode reader (Thermo Scientific). Growth inhibition was calculated as follows (Williams et al. 2012): -

$$\% \text{ GIA} = 100\% - \left\{ \frac{\text{OD of test sample} - \text{OD of uninfected RBCs}}{\text{OD of negative control} - \text{OD of uninfected RBCs}} \times 100 \right\}$$

2.3.5.2 Isolation of polymorphonuclear (PMN) leukocytes from whole blood

Whole blood was collected in EDTA vacutainer tubes and layered over polymorphprep™ (Axis-Shield Diagnostics) in a sterile 15ml falcon tube at a ratio of 1:1. The tubes were centrifuged at 500 x g without brakes for 40mins at room temperature. The layer containing PMNs was carefully aspirated and transferred to a 15ml falcon tube containing 10ml of ice-

cold sterile PMN buffer (0.1% bovine serum albumin (BSA) and 1% D-(+)-Glucose Hybri Max (Sigma) in hanks buffered saline solution). The cells were pelleted at 500 x g for 10mins at 4°C and resuspended in 10ml ice cold 0.2% sodium chloride for 30secs to lyse residual RBCs, followed by addition of 10ml ice-cold 1.6% sodium chloride. After a final spin at 250 x g for 6 mins at 4°C, the cell pellet was resuspended in 0.5ml PMN buffer. Thereafter, 15µl of PMN suspension diluted 1:10 in trypan blue was transferred onto a FastRead 102™ (Immune systems Ltd) disposable hemocytometer and the concentration of isolated PMNs estimated as follows (<http://fastread.co.uk>): -

$$\text{No. of cells/ml} = \text{No. of viable cells in 16 squares} \times 10^4 \times 10(\text{dilution factor})$$

The percentage of neutrophils amongst the PMNs was estimated by smearing 5µl of PMN suspension on a glass slide, fixing in methanol for 10 seconds, staining with 5% Giemsa for 10mins and observing under X100 magnification on a light microscope.

2.3.5.3 Antibody dependent respiratory burst (ADRB) assay

Merozoite lysates comprising of merozoites and debris from lysed schizonts were thawed at room temperature and resuspended by vortexing. Microplate 96 well polystyrene fluorescence Maxisorp surface white (Thermo Scientific Nunc) wells were coated with either 100µl of recombinant protein at 10µg/ml (diluted in 1X PBS) or 100µl of 18.5×10^5 schizonts/ml lysate and incubated overnight at room temperature. The wells were washed three times with 200µl of 1X PBS and blocked with 200µl blocker™ casein in PBS (Thermo scientific) for 1 hour at room temperature. One hundred microlitres of test serum diluted 1:50 in 1XPBS was added to individual wells in duplicate and incubated for 1 hour at 37°C. The wells were washed three times with 200µl of 1X PBS followed by addition of 50µl of 4-Aminophthalhydrazide (Sigma)

at 0.04mg/ml. Thereafter, 50µl of human neutrophils (prepared as described in section 2.3.5.2) diluted to 5×10^5 PMNs/well in PMN buffer were added to individual wells and the plates immediately read on a Varioskan flash multimode reader (Thermo Scientific). Chemiluminescence readings from each well were obtained for one second every minute over an hours' duration.

2.4 Cohort description

Following are details of the characteristics of the Kilifi Birth Cohort (KBC) used to study the targets and mechanisms associated with protection against severe malaria. Details of the cohorts used for the clinical endpoint of uncomplicated malaria are described in Chapter Four.

2.4.1 General characteristics of the KBC cohort

A total of 5,949 infants were recruited between 2001 and 2008 either at the Kilifi District Hospital's maternity ward (4,229 (71.1%)) or at the maternal and child health clinic (1678 (28.2%)) (Figure 2.2). Recruitment information of 42 (0.7%) infants was not entered into the database. 842 (14%) infants were not linked to the KHDSS register (Fig 2.2) because matching of KBC recruits to the population register began much later in April 2002 (Scott et al. 2012). The mean maternal age was 26.7 years (range 17 – 59). The median number of siblings was 2 (range 0-11). A total of 20, 219 cohort visits were recorded during the study period. A total of 222 children were selected for the matched case-control study comprising of 61 well-defined severe malaria cases and 161 controls. The baseline characteristics of the study participants are summarized in Table 2.2.

2.4.2 Summary of severe malaria cases and controls

A total of 93 children fulfilled the criteria for well-defined severe malaria (Marsh et al. 1995). Sixty one cases had three-monthly follow-up serum samples available (Figure 2.2). The distribution of clinical symptoms of severe malaria amongst the 61 cases is summarized in Figure 2.3. The median age of index cases at the time of admission was 14.8 months (IQR 7.5 – 24.7) and four cases had fatal outcomes. The median parasitemia was 26,691 parasites/ μ l (IQR 4,020– 260,000). The localities within the KHDSS from which the severe malaria cases were drawn are shown in Figure 2.4.

A total of 161 controls were selected (Figure 2.2), 58 of whom had a history of admission to hospital with uncomplicated malaria (11 (19%)), lower respiratory tract infections (13 (22%)), gastroenteritis (12 (21%)) or other illnesses (22 (38%)) such as neonatal sepsis, bronchiolitis, neonatal jaundice, burns, malnutrition and febrile convulsions.

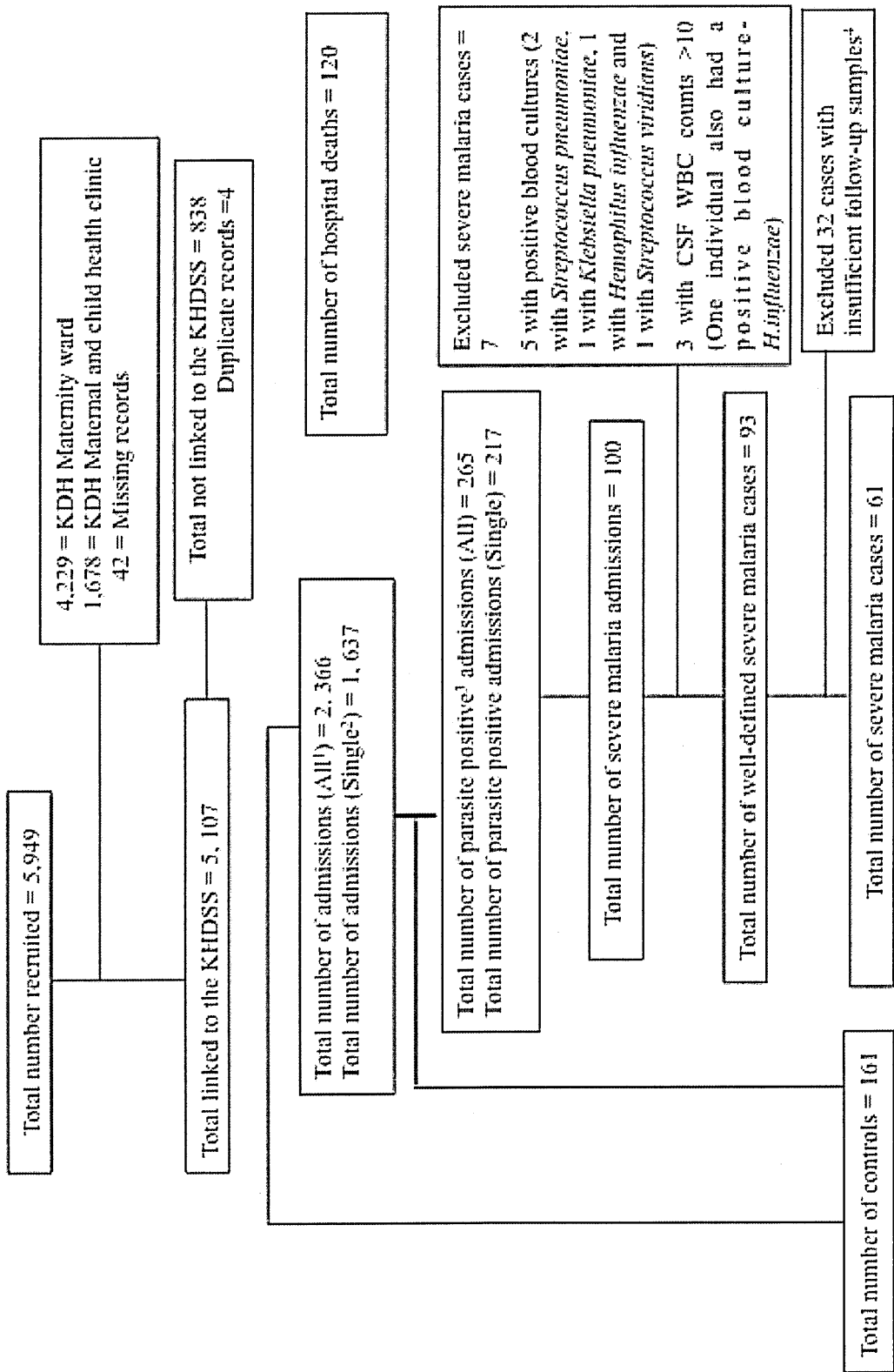


Fig 2.2: A schematic diagram of the Kilifi Birth Cohort illustrating the selection of cases and controls for the matched case-control study.

¹ Total number of admissions during the follow-up period. Some children were admitted to hospital more than once.

² Unique admissions during the follow-up period.

³ Parasite positive refers to presence of detectable parasites by microscopy.

⁴ Thirty two cases were excluded because their 3-monthly follow-up visit serum samples were depleted.

Table 2.2: Baseline characteristics of selected cases and controls illustrating the success of matching.

	Cases (N=61)	Controls (N=161)	P-value
Sex, female/N (%)	31/61 (50.8)	78/161 (48.4)	0.76
No. of visit samples, mean (range)	4.36 (1-8)	4.49 (1-9)	0.49
Age at the time of index case admission, median age in months (IQR)	14.83 (7.5 – 24.53)	16.36 (7.3 – 24.8)	0.81
Proportion parasite positive ^a during visits, n/N (%)	21/170 (12)	36/458 (7.8)	0.08
Proportion of individuals with fever during visits, n/N (%)	14/153 (9.1)	37/443 (8.3)	0.90
Proportion of individuals with fever and parasites during visits, n/N (%)	5/165 (3%)	12/427 (2.8%)	0.95

^aParasite positive either by microscopy or PCR

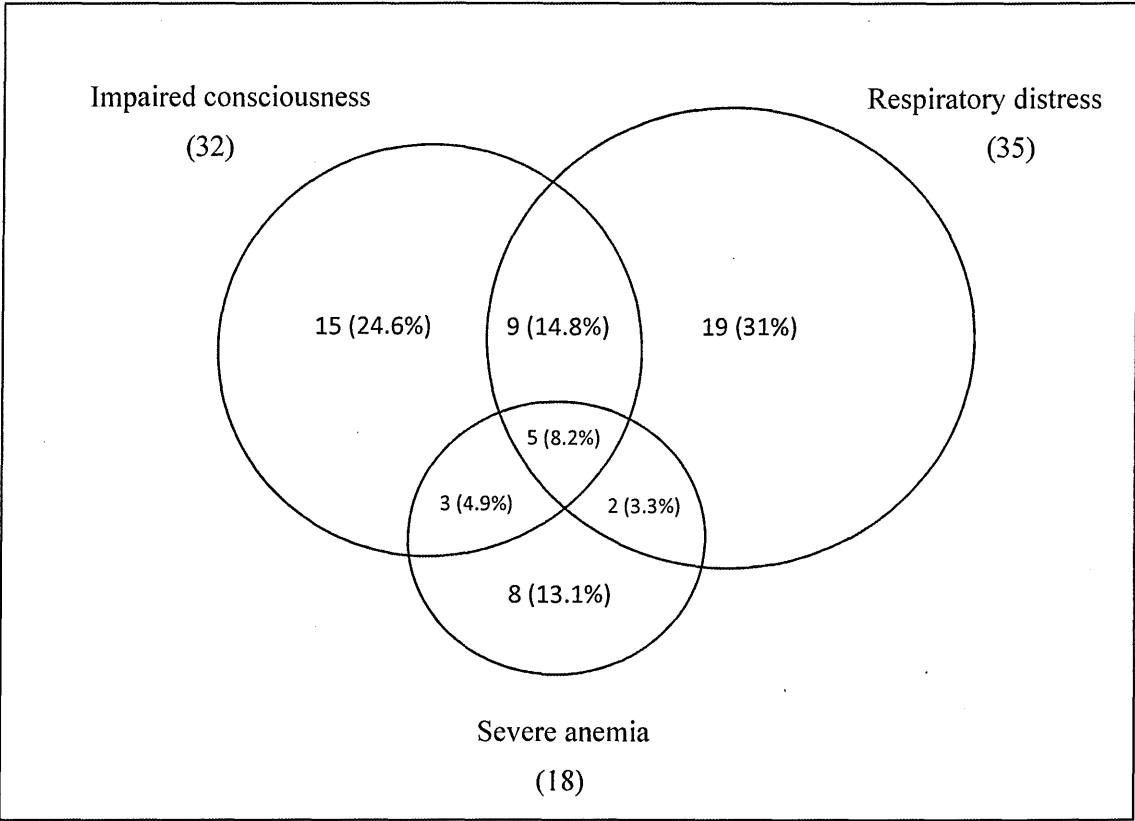


Figure 2.3: The prevalence and overlap of the major severe malaria symptoms amongst the cases.

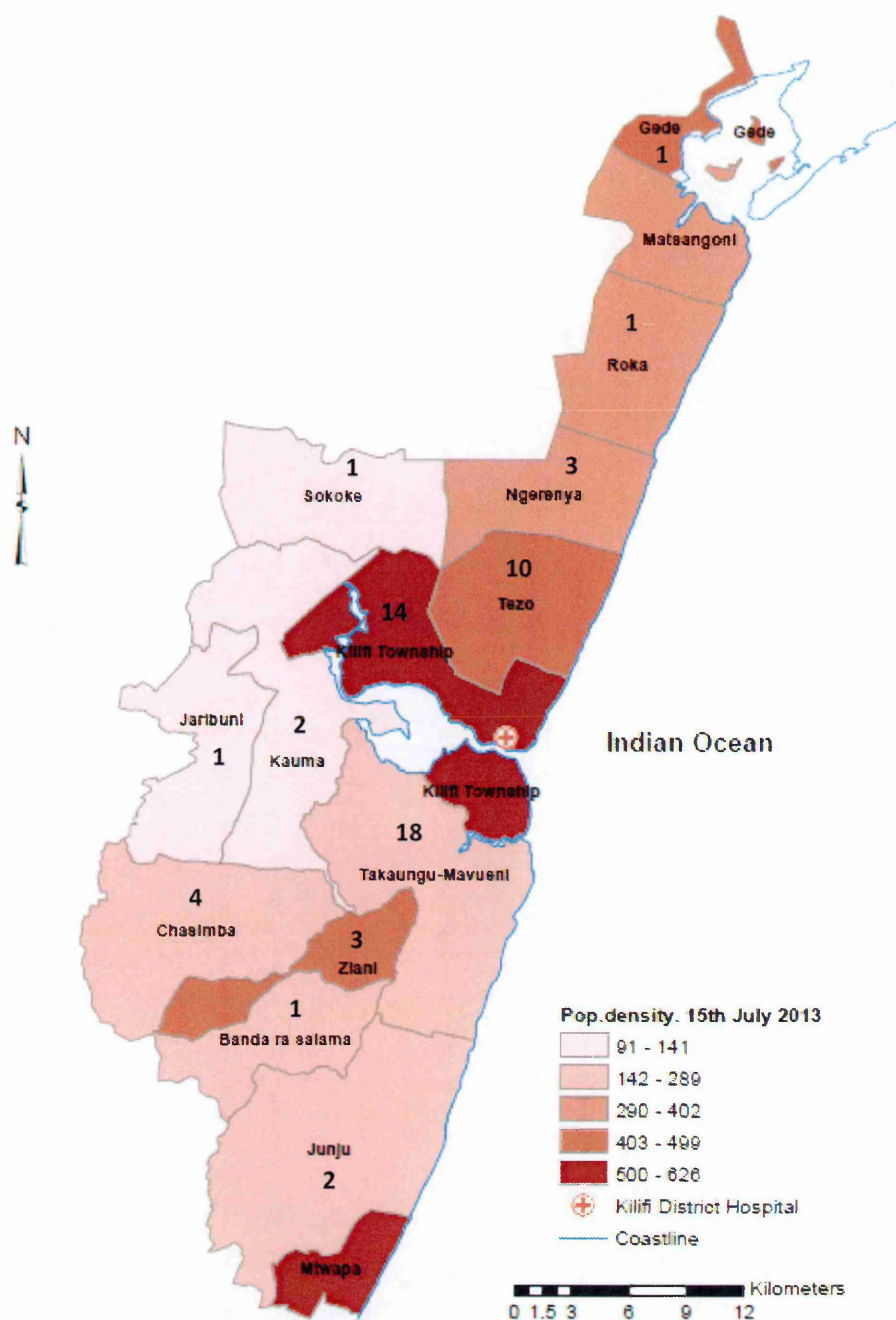


Figure 2.4: Map of KHDSS showing the number of KBC severe malaria cases recorded from each administrative location during the follow-up period (map kindly provided by Mark Otiende, KHDSS data manager).

CHAPTER THREE

Comparison of ELISA methods for quantifying malaria-specific antibody amounts

3.0 Introduction

Quantification of malaria-specific antibodies is important for determining the level of antibodies that may be associated with protection from episodes of malaria in immunoepidemiological studies and for evaluating the immunogenicity of malaria vaccines. Enzyme Linked Immunosorbent Assay (ELISA) based approaches have been widely used to quantify antibodies and have replaced traditional methods such as the radioimmunoassay. The ELISA method was developed simultaneously and independently in 1971 by Engvall and Perlmann (Engvall and Perlmann 1971) and by van Weemen and Schuurs (Van Weemen and Schuurs 1971). Although this method is reliable, sensitive, reproducible and fairly low cost, considerable differences exist between studies in the reporting of the magnitude of responses.

In the field of malaria, some studies report ELISA optical density (OD) values as a measure of the magnitude of responses (Osier et al. 2008, Kinyanjui et al. 2007, Agak et al. 2008, Polley et al. 2006, Polley et al. 2004) whose accuracy is limited by the non-linear relationship observed between OD values and antibody concentration outside specific ranges of a standard curve. In my studies, the linear range of a standard curve is limited to about one order of magnitude, ranging between an OD of 0.2 – 2, while the actual concentration in a sample could range over five to six orders of magnitude. This is a concern when measuring antibody titers of individuals living in high malaria transmission settings or those mounting effective responses after vaccination, since such individuals would induce anti-merozoite antibody levels that fall above the linear range of a standard curve with the possibility that their antibody titres would be underestimated. Above this

range, a two-fold difference in OD readings does not equal to a two-fold difference in the absolute antibody amount (Engvall and Perlmann 1972, Plested, Coull and Gidney 2003).

To overcome this limitation, the determination of end point titers from dilution curves of individual sera has been adopted in some studies (Dutta et al. 2009, Dodoo et al. 2011). Here, titers are defined as the highest dilution of serum that gives an OD reading above a specified cutoff. Some studies employ a cutoff of two or three standard deviations of the mean of the negative control or background (Stowers et al. 2001, Genton et al. 2007) or an arbitrary OD value (Miura et al. 2008a, Dutta et al. 2009). Given that this method requires a full dilution curve of individual sera, which is labor-intensive and requires a lot of reagents, it is not suited for large cohort studies. An attempt has been made to standardize end point titer cutoffs by employing statistical methods (Frey, Di Canzio and Zurakowski 1998).

Other studies have reported relative antibody units with reference to the units assigned to a pool of sera from malaria-exposed individuals (Dodoo et al. 2000, Corran et al. 2008, Nebie et al. 2008b) or a standard commercial reagent (Nebie et al. 2008a, Lusingu et al. 2005, Sirima et al. 2007, Sirima et al. 2009). Antibody amounts in test sera are then reported as arbitrary units since the actual antigen-specific amount in the standard is unknown and, thus far, there is a lack of malaria-specific reference reagents (with known antigen-specific antibody concentrations) to determine absolute antigen-specific antibody amounts. In a study by Yoon *et al.*, the development of an MSP-1₄₂ specific reference standard of known absolute concentration has been described (Yoon et al. 2005) and employed in the quantification of MSP-1₄₂-specific antibodies (Ogutu et al. 2009). However, this is one of hundreds of merozoite antigens and a similar standard would be needed for all the remaining antigens and for each species to be studied.

In the absence of a known antigen-specific reference reagent to determine absolute antibody concentrations, Miura *et al.* have demonstrated that arbitrary units can be

converted into absolute concentrations (in $\mu\text{g/ml}$) using a mathematical conversion factor (Miura et al. 2009). Although this approach is mainly limited by the quantity of antigen and serum that is required to perform antigen-specific antibody affinity purification, it has been adopted by several studies assessing vaccine-induced immunity against MSP-1 and AMA1 (Sheehy et al. 2011, Sheehy et al. 2012a, Ellis et al. 2010, Ellis et al. 2012, Pierce et al. 2010). Another method of antibody quantification known as the calibration-free concentration analysis (CFCA) has been described and utilized for absolute quantification of merozoite-specific antibody concentrations (Williams et al. 2012). CFCA measures the rate of binding of specific antibodies to their target antigens by surface plasmon resonance (SPR). A fully quantitative and standardized measure of antibody responses would allow better comparison of data from different studies.

The heterogeneity in the methods used to quantify antibodies to malaria antigens by different studies has hindered accurate comparison of findings across studies and the standardization of methods of expressing ELISA data is essential in this regard.

3.1 Rationale

In this chapter, I sought to determine the best method to adopt for quantification of antibodies against *P. falciparum* merozoite antigens that could replace the use of OD values (limitations outlined above) which have previously been widely applied as a measure of the magnitude of responses in several cohorts in Kilifi (Agak et al. 2008, Osier et al. 2008, Kinyanjui et al. 2007, Polley et al. 2004, Polley et al. 2006). Towards this end, I tested a quantification method outlined by Miura *et al*, which reliably estimates arbitrary units using a single dilution of sera (Miura et al. 2008a). Titres of test sera are determined from a serially diluted hyperimmune serum pool and defined as the reciprocal dilution that gives an OD equal to 1.0. Titres obtained by this method are identical to those derived from a full dilution curve of individual samples at this cutoff. I compared antibody titres

obtain by this method with those obtained by interpolating relative amounts from a standard curve derived from a purified immunoglobulin pool assigned an arbitrary concentration of 50mg/ml. Evaluating the best quantification method would enable comparison of data from different cohorts in Kilifi and in other settings if the same method were applied.

3.2 Objective

- To evaluate and compare two ELISA methods for the quantification of malaria-specific antibodies.

3.3 Methods

3.3.1 Recombinant antigens and standard ELISA

Antibody titres against AMA1, MSP-2, MSP-3 and MSP-1₁₉ OD were evaluated using a standard ELISA protocol as outlined in section 2.3.2.1.

3.3.2 Semi-immune adult sera

Relative antibody amounts were interpolated from standard curves generated from two independent serum pools prepared from malaria-exposed adults who are known to be semi-immune to clinical episodes of malaria.

3.3.2.1 Pool of sera from malaria exposed Kilifi adults (PHIS)

Serum samples collected between 1995 and 2008 during annual cross-sectional surveys from healthy adults resident in Kilifi County were pooled and aliquoted into 1ml vials. The

samples were stored at -80°C. Eleven doubling dilutions of the pooled serum were used in the ELISA assay to generate a standard curve.

3.3.2.2 Malaria Immune Globulin (MIG)

A commercially purified immunoglobulin reagent of known concentration (50mg/ml) was also provided for use as a malaria-specific reference standard (Central Laboratory Blood Transfusion Service SRC, Switzerland). The preparation contains immunoglobulins (98% IgG) purified from a plasma pool of eight hundred and thirty four healthy Malawian adults and was originally manufactured to test its potential use as an adjunct therapy to quinine in the treatment of cerebral malaria (Taylor et al. 1992). The absolute concentration of antibodies to specific malaria antigens in this reagent has thus far not been determined and therefore this pool was assigned an arbitrary concentration of 50mg/ml.

3.3.3 Assigning antibody units to the pooled semi-immune serum (PHIS) and MIG

Eleven two-fold serial dilutions of PHIS were prepared, starting at 1:250 to 1:256,000. ELISA OD values were determined at each dilution against AMA1, MSP-3, MSP-2 and MSP-1₁₉. The OD values were plotted against the reciprocal dilution to generate a standard curve fitted using the four-parameter logistic function in GraphPad Prism software, version 6.0 (GraphPad Software, San Diego, CA). Antibody units for each antigen tested were assigned to PHIS as the reciprocal dilution giving an OD₄₉₂ equal to 1. These were invariably used as the antibody units contained in the semi-immune serum pool against these antigens in subsequent assays.

The Malaria Immune Globulin (Central Laboratory Blood Transfusion Service SRC, Switzerland) was commercially prepared and packaged in 50mg/ml vials. The absolute antigen-specific antibody concentrations contained in MIG has not been determined thus

far and it was therefore assumed to be 50mg/ml for every antigen tested to enable interpolation of relative antibody amounts from a standard curve.

3.3.4 Determining antibody units of test samples from a standard curve of PHIS and MIG

One hundred and thirty one adult serum samples were screened for the range of ELISA OD values obtained at a serum dilution of 1:1000 against four merozoite antigens using a standard ELISA protocol. Serum samples with high OD values ($OD > 2.5$), intermediate OD values ($OD 1 - 2.5$) and low OD values ($OD < 1$) were selected for each antigen tested to capture the range of OD values obtained from a standard ELISA in our setting. Serial dilutions of PHIS and MIG were included for every antigen to generate a standard curve which was used to convert ELISA OD values to relative antibody units.

3.3.5 End point titration of individual test sera

Twelve two-fold serial dilutions of individual test sera were prepared starting at 1:1,000 to 1:1,024,000 dilution. The samples were tested for reactivity to AMA1, MSP-3, MSP-2 and MSP-1₁₉. Individual curves of test samples were fitted using the four-parameter logistic function, as described above. The end point titer for each sample was defined as the reciprocal dilution that gave an OD_{492} equal to 1.

3.3.6 Statistical analysis

Spearman correlation coefficients were calculated using GraphPad prism version 6.0 (GraphPad software, San Diego, California, USA). A four-parameter logistic function was

used to fit standard curves in GraphPad Prism software, version 6.0 (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 Assigning antibody units to PHIS and MIG

The antibody units assigned to PHIS were 11700AU, 3070AU, 3540AU and 3250AU for AMA1, MSP-3, MSP-2 and MSP-1₁₉ respectively (Figure 3.1). These were invariably used as the total antibody concentrations of the hyperimmune pools for these antigens in subsequent assays. The antibody concentration of MIG was assumed to be 50mg/ml for all antigens.

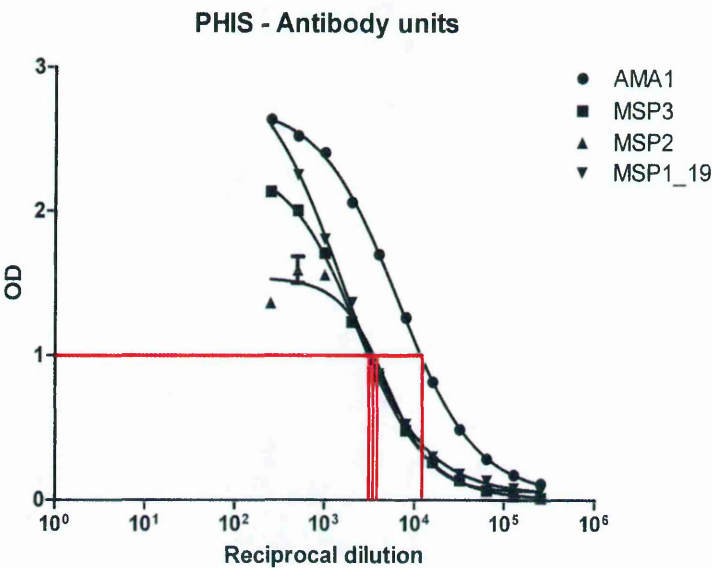


Figure 3.1 Standard curves of a semi-immune serum pool (PHIS) against AMA-1, MSP-3, MSP-2 and MSP-1₁₉. Antibody units contained in the pool for each antigen were determined as the reciprocal dilution that gave an OD₄₉₂ equal to 1.0.

3.4.2 End point titration

To ensure that the quantification method was accurate across the range of ELISA OD values obtained in our setting, twenty two adult samples with low, intermediate or high OD values were selected for each antigen. Test sera were diluted 1:1000. Thereafter, full dilution curves were generated for the 22 adult samples. All samples exhibited the expected sigmoid shape (Figure 3.2). The end point titer was defined as the reciprocal dilution that gave an OD₄₉₂ equal to 1. Titers for 4 (18%), 9 (41%), 10 (45%), and 12 (55%) samples tested against AMA1, MSP-3, MSP-2 and MSP-1₁₉ respectively could not be determined using this method because the OD observed at the maximum concentration was below the end point titer cutoff (defined as OD₄₉₂ =1).

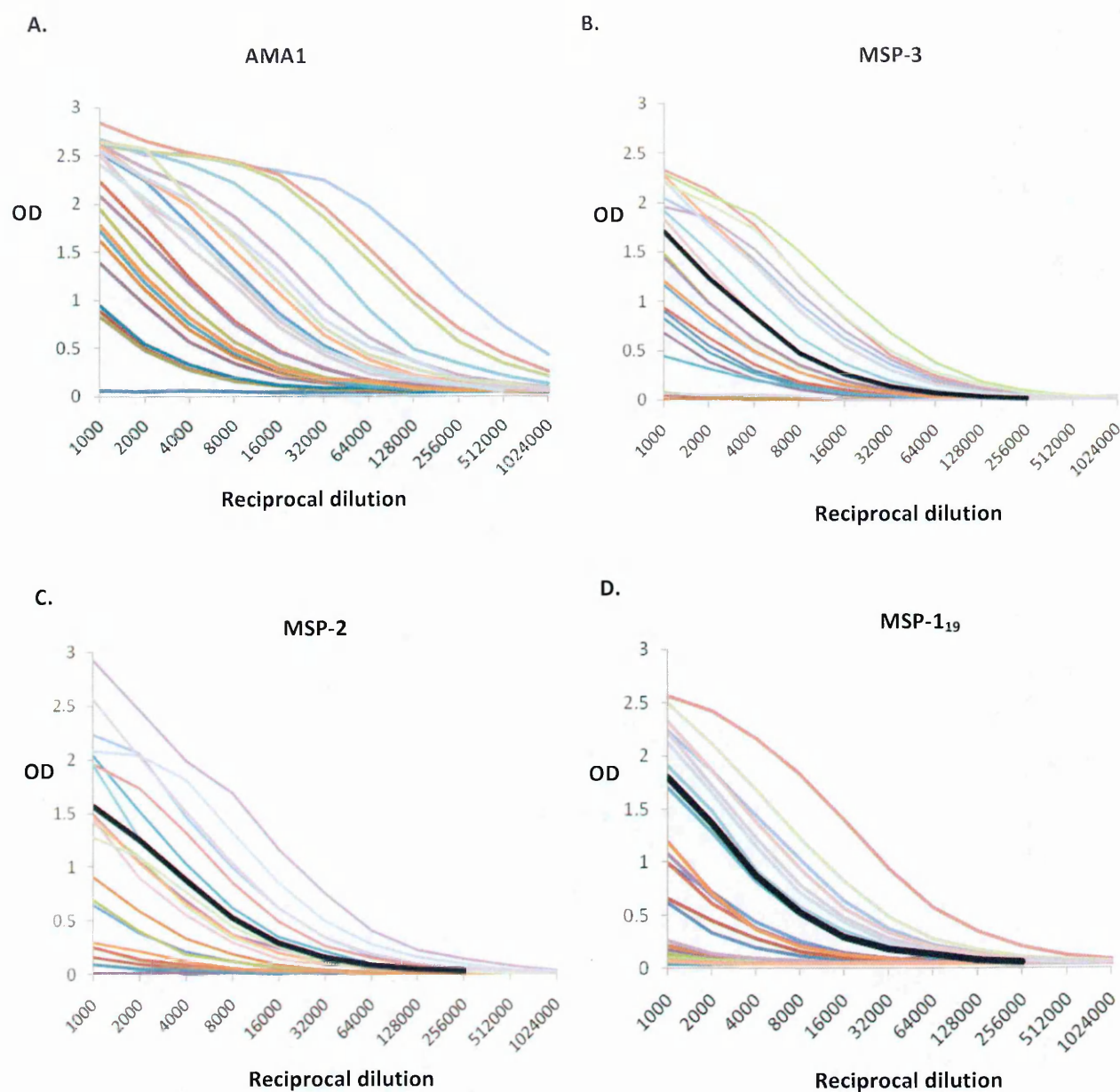


Figure 3.2: End point titration curves of 22 sera tested against (A) AMA-1 (B) MSP-3 (C) MSP-2 and (D) MSP-1₁₉. Two-fold serial dilutions were generated for every sample starting at 1:1000 to 1:1024000. A curve of each sample was fitted using the four-parameter hyperbolic function. End point titers were defined as reciprocal dilution giving an OD₄₉₂ equal to 1.0.

3.4.3 Comparison of antibody units derived from a single dilution (1:1000) versus a full dilution curve

Based on the method described by Miura *et al*, antibody amounts extrapolated from the serially diluted standard (PHIS) at a single dilution are similar to those obtained using the end point titer method (Miura et al. 2008a). End point titers are defined as the reciprocal dilution that gives an OD₄₉₂ equal to 1. The correlations between amounts derived from a single serum dilution of 1:1000 were compared to those obtained from a full dilution curve (1:1000 to 1:1024000) of individual samples. For all antigens tested, there was a significant positive correlation between end point titers obtained by both methods (Spearman correlation coefficient of 0.987, 0.968, 0.936 and 0.991 for AMA1, MSP-3, MSP-2 and MSP-1₁₉, respectively) (Figure 3.3). However, there was a large difference in antibody units assigned by both methods in 13 (59%), 13 (59%), 12 (54%) and 10 (45%) samples tested against AMA1, MSP-3, MSP-2 and MSP-1₁₉, respectively, for samples that had an OD₄₉₂ greater than 2.07, 1.95, 1.91 and 2.12, or less than 0.05, 0.245, 0.07 and 0.101, respectively. These OD₄₉₂ values thus define the limits within which reliable estimation of antibody amounts from a standard curve at a serum dilution of 1:1000 could be obtained. Since more than half of the samples tested were assigned incorrect antibody amounts at this dilution, the determination of a single dilution that gave OD values closest to 1 was essential.

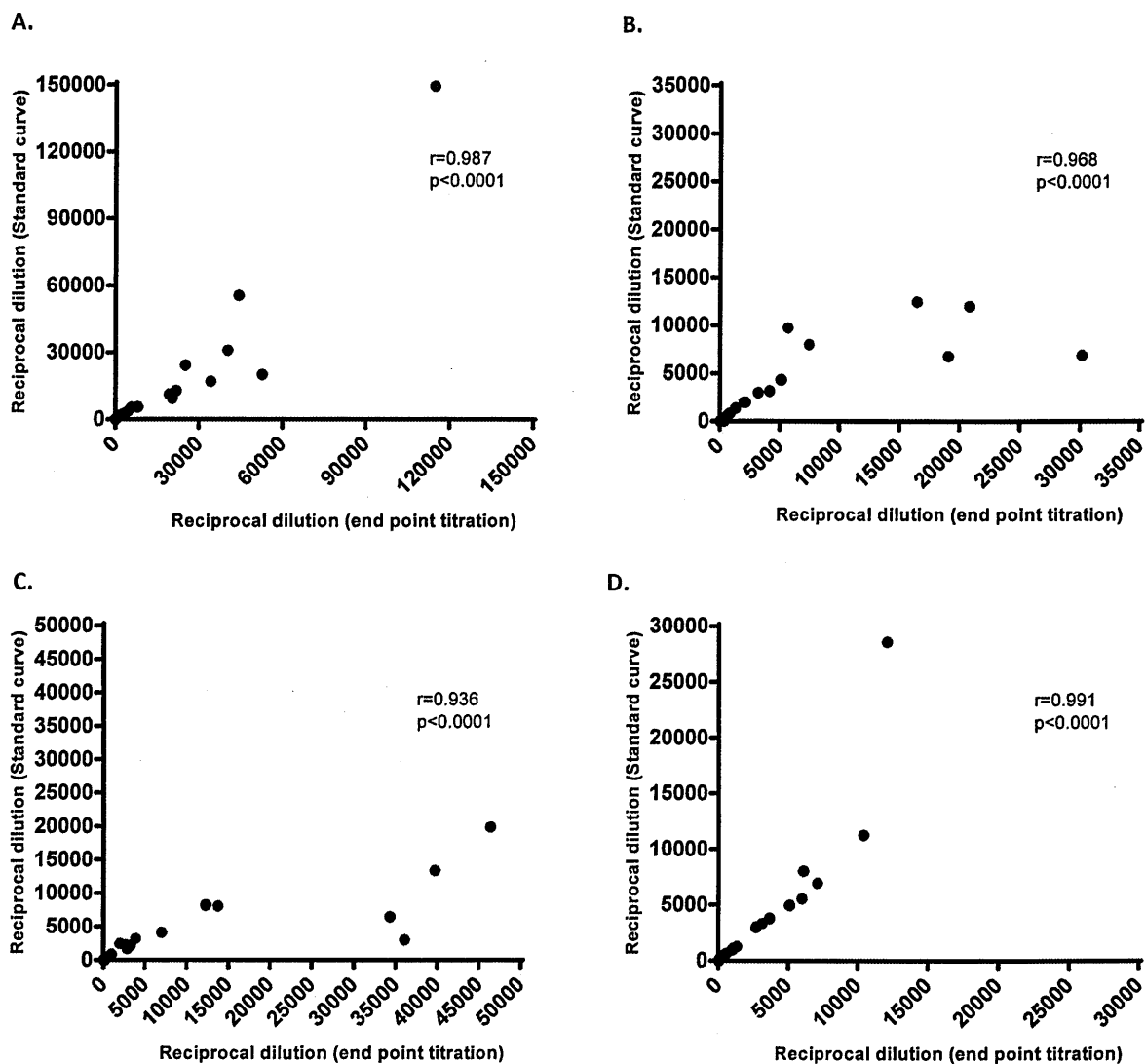


Figure 3.3. Comparison of end point titers calculated from a single dilution point (1:1000) versus a full dilution curve of individual samples. The end point titre was defined as the OD₄₉₂ that gave an OD equal to 1. Representative graphs of responses to (A) AMA1 (B) MSP-3 (C) MSP-2 and (D) MSP1-19 are shown.

3.4.4 Determining an optimal single serum dilution for estimating accurate antibody concentrations from a standard curve

Having determined that a single dilution could accurately reflect the titres measured using a full end point titration, I then sought to determine what that optimal serum dilution ought to be. To do this, end point titers derived from full dilution curves of individual test sera were plotted against titers interpolated from the standard PHIS curve at five doubling serum dilutions (1:2000 – 1:32000) and the correlation coefficients for each pair compared.

There was a significant positive correlation between the end point titers derived from full dilution curves and from single dilutions at all the serial dilutions tested for all antigens (Spearman correlation coefficient >0.9 ; $p<0.0001$), (Figure 3.4.1 – 3.4.4). There was an increase in the correlation coefficient with increasing serum dilution for all antigens tested (Figure 3.4.1 – 3.4.4). Based on these findings, a dilution of 1:16000 was selected as the optimal serum dilution for determining antibody concentrations from the standard PHIS curve as it gave the highest correlation coefficient for all antigens tested (Spearman correlation coefficient = 1, 0.998, 0.992 and 0.991 for AMA1, MSP-1₁₉, MSP-2 and MSP-3, respectively).

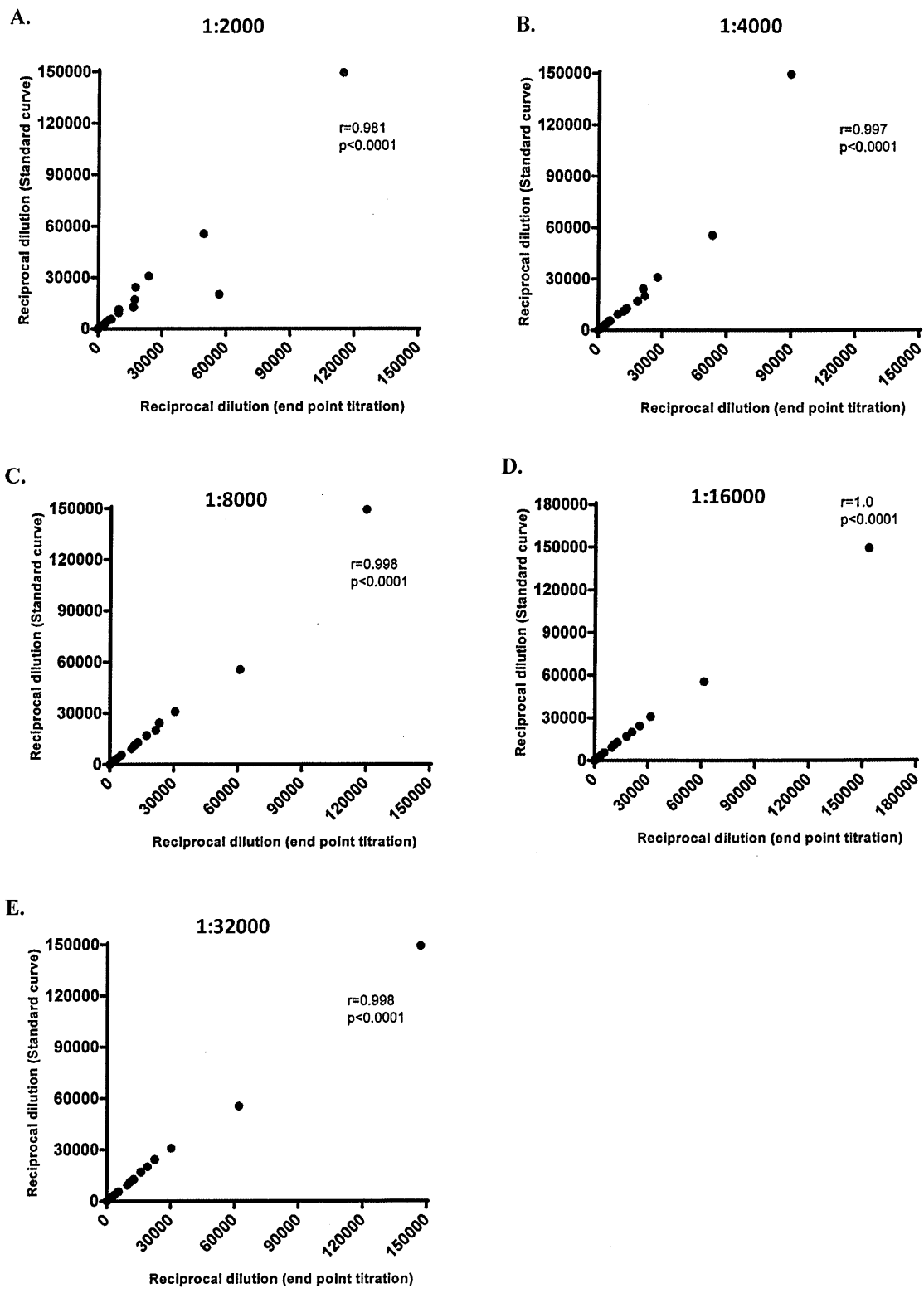


Figure 3.4.1: AMA-1 end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis). Serum samples were diluted at (A) 1:2000 (B) 1:4000 (C) 1:8000 (D) 1:16000 and (E) 1:32000 and titers interpolated from the standard PHIS curve. End point titres were defined as the reciprocal dilution that gave an OD₄₉₂ equal to 1.

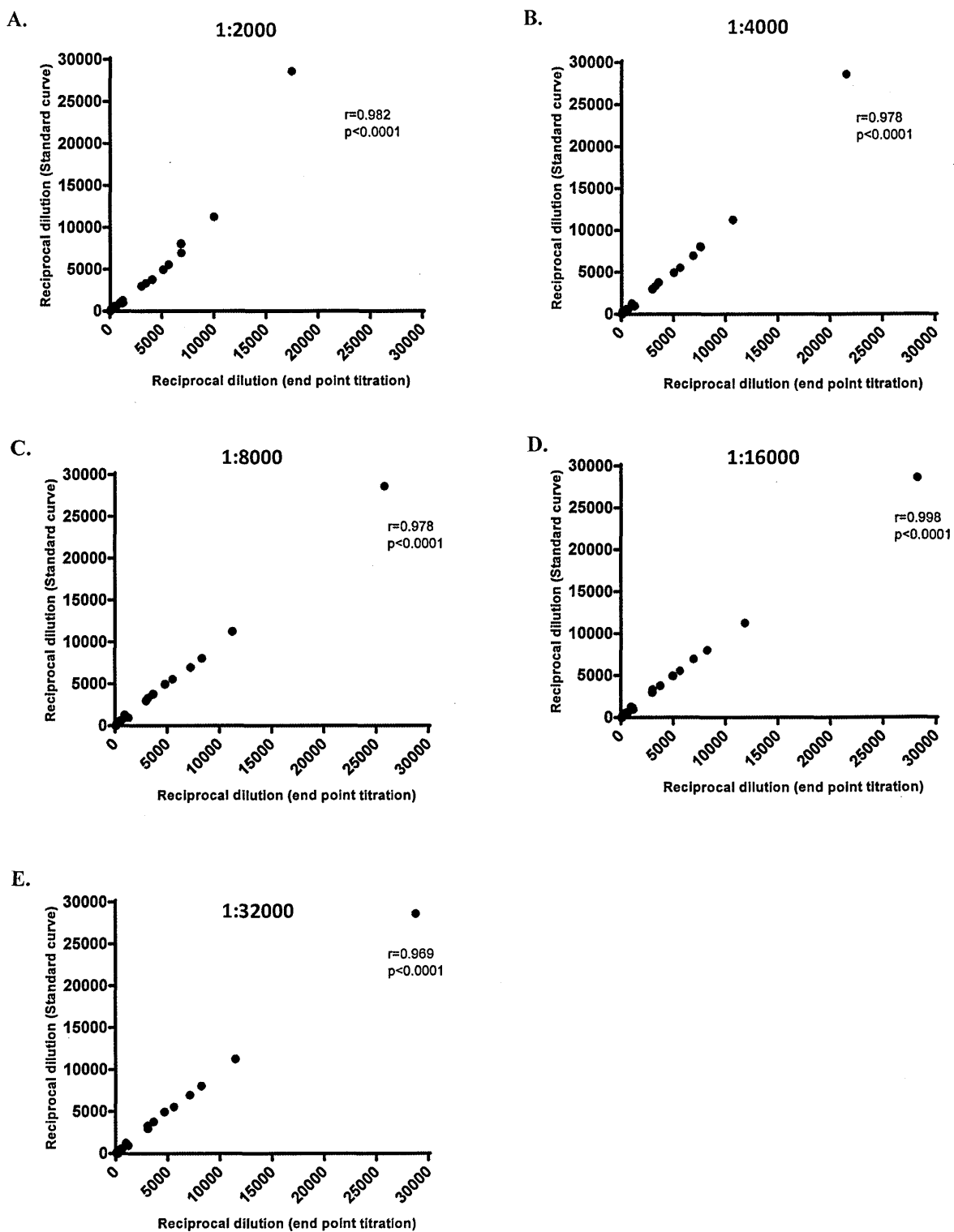


Figure 3.4.2: MSP-1₁₉ end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis). Serum samples were diluted at (A) 1:2000 (B) 1:4000 (C) 1:8000 (D) 1:16000 and (E) 1:32000 and titers interpolated from the standard PHIS curve. End point titres were defined as the reciprocal dilution that gave an OD₄₉₂ equal to 1.

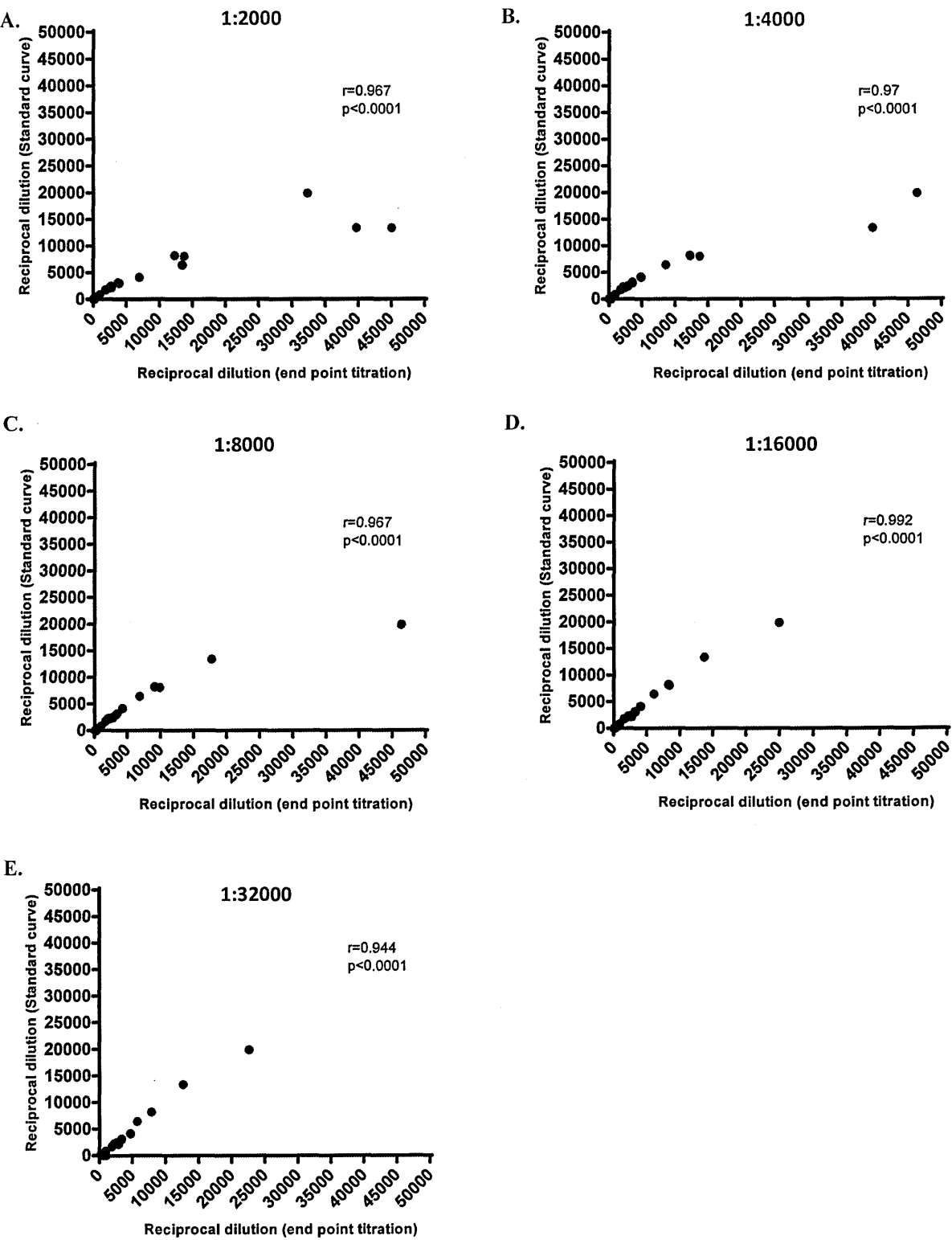


Figure 3.4.3: MSP-2 end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis). Serum samples were diluted at (A) 1:2000 (B) 1:4000 (C) 1:8000 (D) 1:16000 and (E) 1:32000 and titers interpolated from the standard PHIS curve. End point titres were defined as the reciprocal dilution that gave an OD₄₉₂ equal to 1.

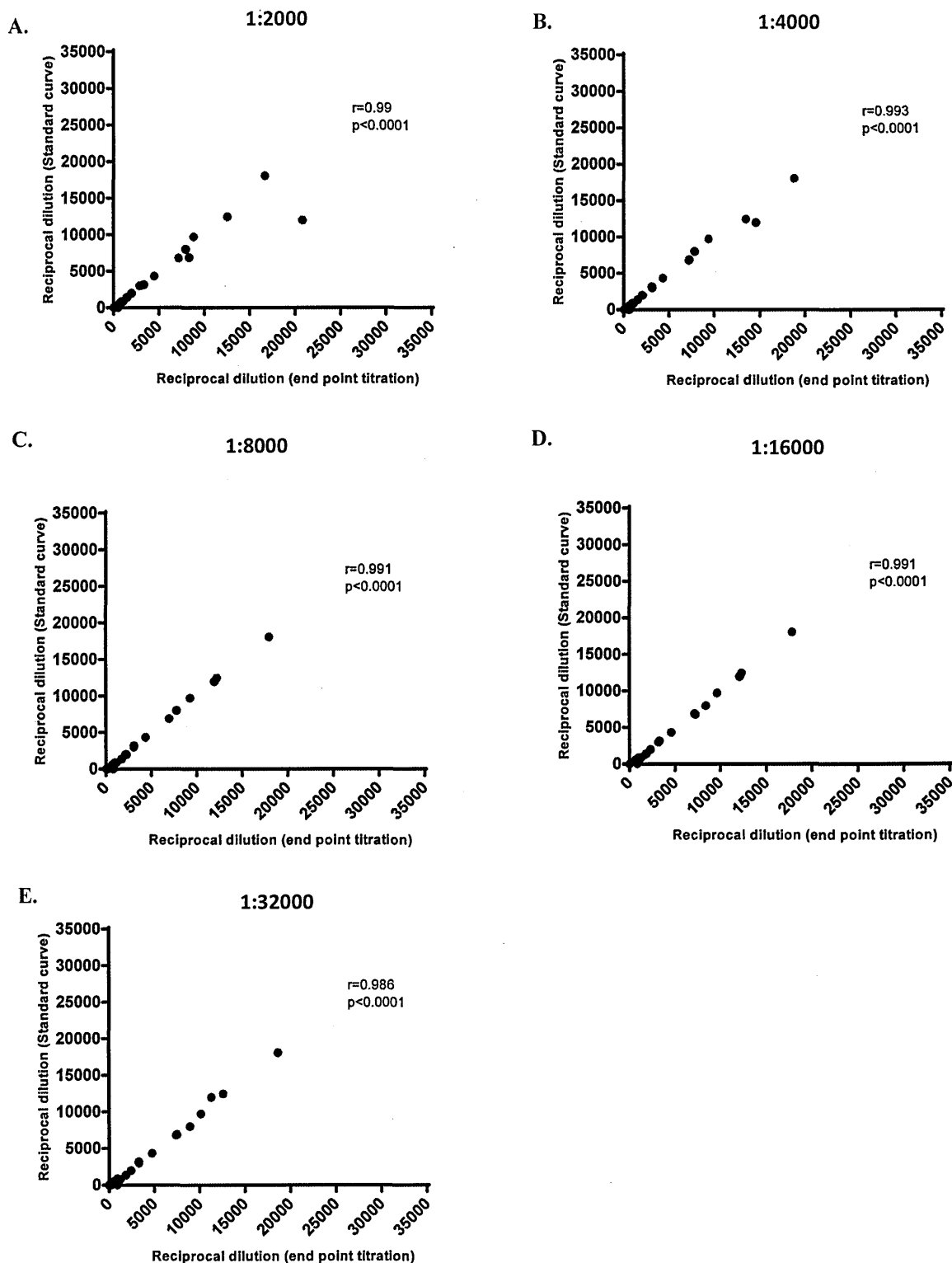


Figure 3.4.4: MSP-3 end point antibody titers estimated from individual full dilution curves of test sera (x-axis) versus titers derived from a standard curve of PHIS (y-axis). Serum samples were diluted at (A) 1:2000 (B) 1:4000 (C) 1:8000 (D) 1:16000 and (E) 1:32000 and titers interpolated from the standard PHIS curve. End point titres were defined as the reciprocal dilution that gave an OD₄₉₂ equal to 1.

3.4.5. Determining relative antibody amounts from a standard curve of MIG

Having optimized the method of accurately determining arbitrary antibody amounts from a standard PHIS curve using a single serum dilution, a purified immunoglobulin pool (MIG) of known concentration was also used to obtain arbitrary antibody concentrations (in $\mu\text{g/ml}$) based on the assumption that the MIG pool contains 50mg/ml arbitrary units of antibody against each antigen. Twelve two-fold serial dilutions of MIG were prepared, starting at 160 $\mu\text{g/ml}$ to 0.078 $\mu\text{g/ml}$ and tested against AMA1, MSP-2 (Dd2 and CH150/9 allelic types), MSP-3 (K1 and 3D7 allelic types), GLURP-R0, GLURP-R2 and MSP-1₁₉ (Figure 3.5).

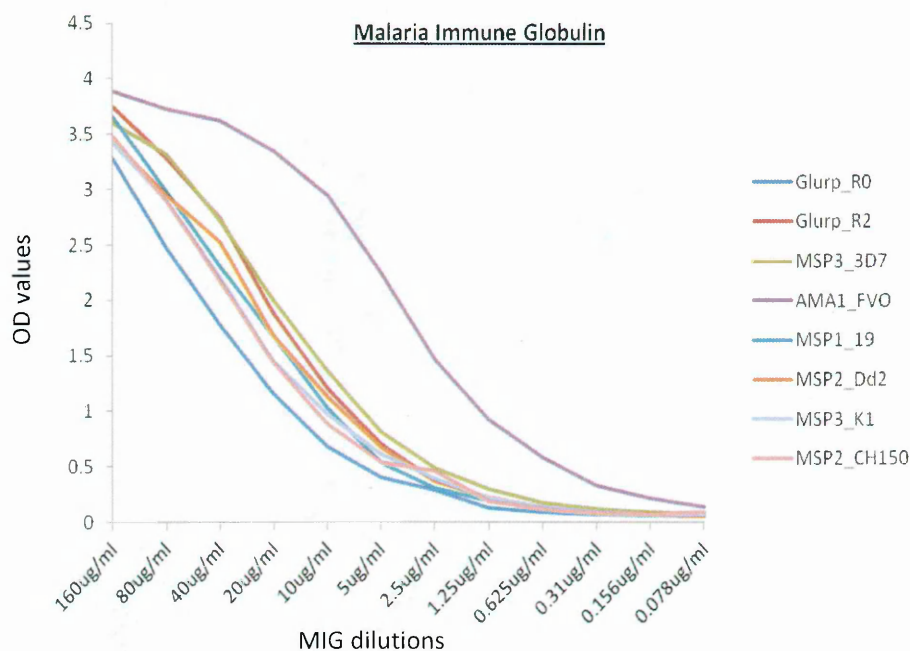


Figure 3.5. Serial dilution curves of MIG tested against eight merozoite antigens. Each line represents a dilution curve for an individual antigen.

A prerequisite for correct estimation of relative antibody concentrations from a standard is the confirmation that the shape of the standard curve is similar to that of individual test samples (Miura, 2008). The dilution curves for MIG were parallel to those of the test samples when tested against AMA-1 and MSP-3 (Figure 3.6); therefore correct relative estimates of concentrations could be determined in this population using MIG as a standard. Thereafter, relative antibody amounts of 22 adult samples were determined from a standard curve of MIG generated using the four-parameter logistic function. Samples were assayed at a single serum dilution of 1:16000.

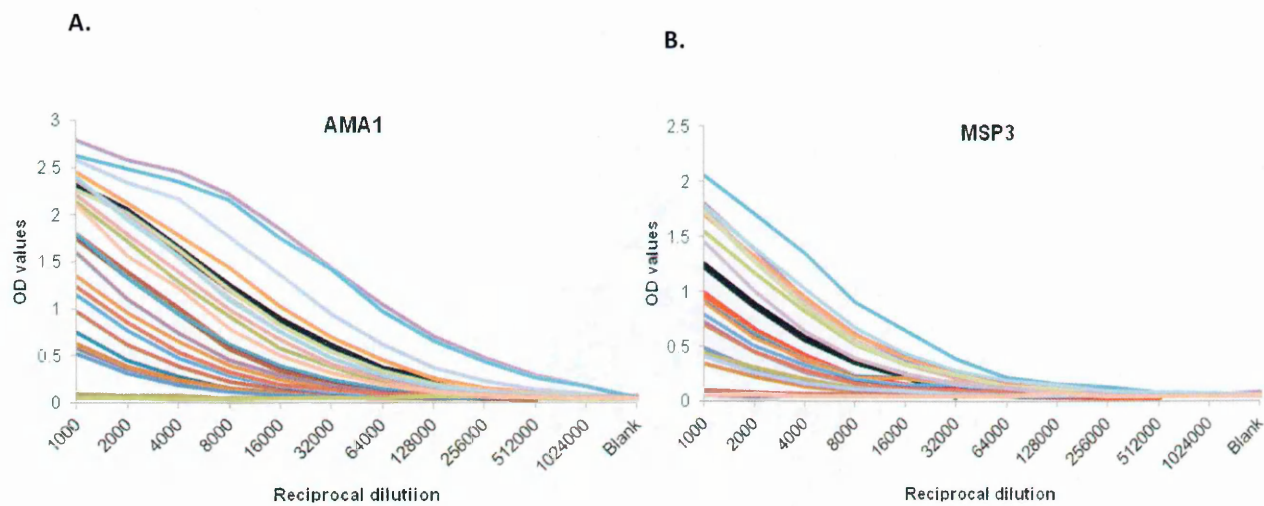


Figure 3.6. Individual dilution curves (1:1000 to 1:1024000) of twenty two adult serum samples and a malaria specific standard reference reagent (MIG) represented as a black bold line. Responses to (A) AMA1 and (B) MSP-3 are shown.

3.4.6. Comparison of antibody amounts derived from a standard curve of PHIS versus MIG

End point titers obtained using a standard curve of PHIS were compared to relative antibody amounts derived from a standard curve of MIG, assuming a concentration of 50mg/ml in the MIG pool. There was a significant positive correlation between results obtained by both approaches for anti-AMA1 and MSP-3 antibodies (spearman correlation coefficient = 0.998 and 0.984; respectively), (Figure 3.7).

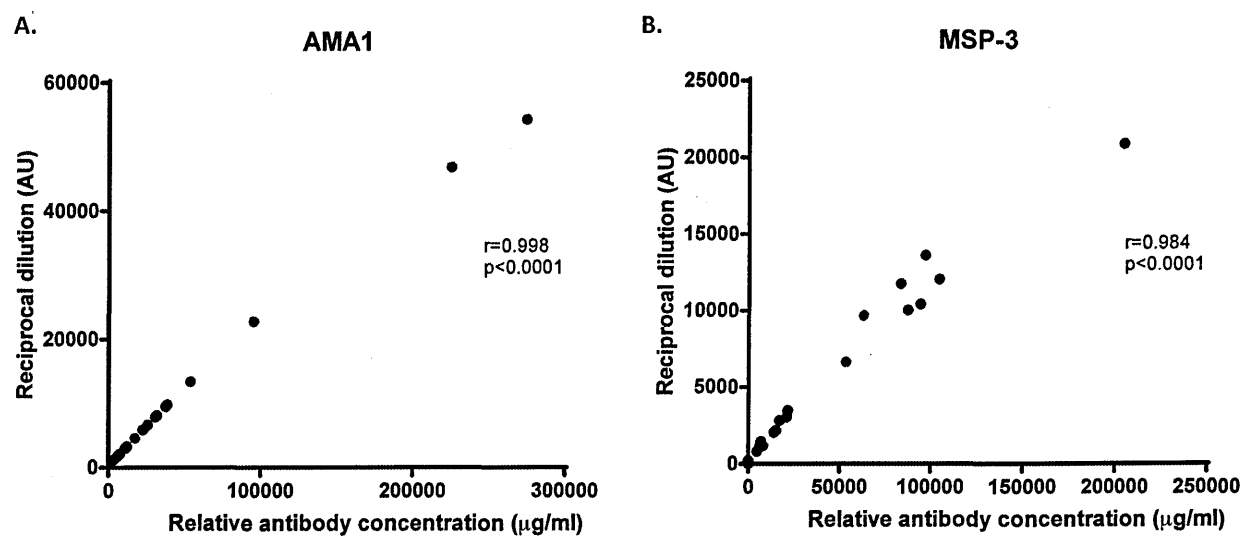


Figure 3.7. Comparison of end point titers derived from a standard curve of PHIS and relative amounts derived from a standard curve of MIG. Representative graphs of responses to (A) AMA1 and (B) MSP-3 are shown.

3.4.7 Reproducibility of the ELISA data

Serum samples (n=22) were tested on two separate days, at an interval of one month, to determine the reproducibility of the ELISA data (end point titers) generated from a standard curve of PHIS. There was a significant positive correlation between antibody amounts obtained on two separate days for AMA1 and MSP-3 (spearman correlation coefficient = 0.985 and 0.971; respectively), (Figure 3.8).

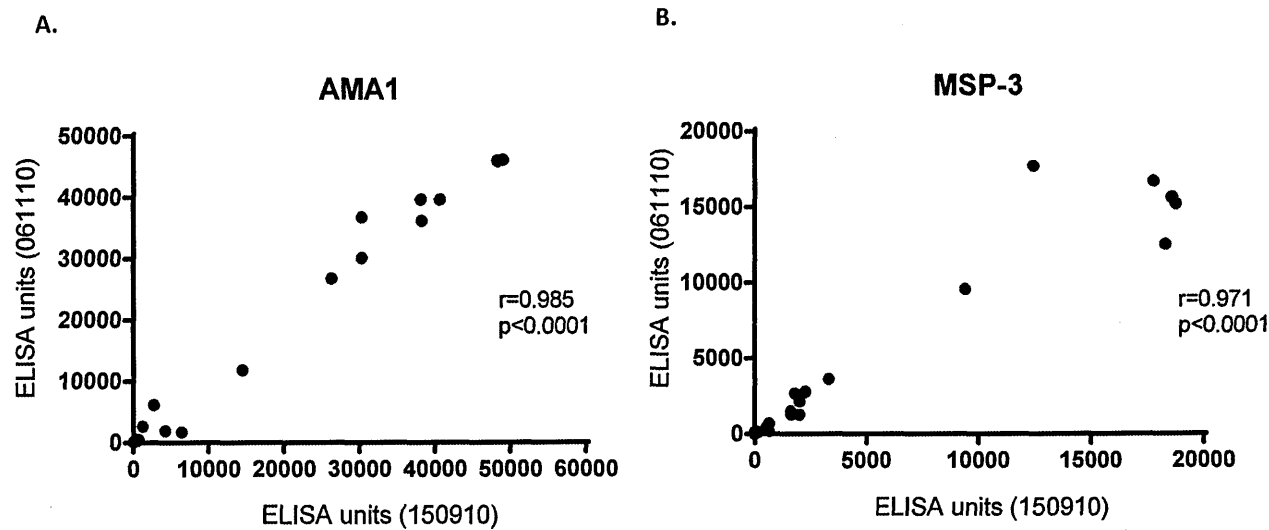


Figure 3.8. Reproducibility of ELISA data. The assays were performed on different days and the antibody amounts interpolated from a standard curve of PHIS. Representative graphs of responses to (A) AMA1 and (B) MSP-3 are shown.

3.5 Discussion

In the absence of a reference reagent of known antigen-specific concentration, to provide an estimate of relative antibody concentrations in test sera, antibody amounts can be quantified relative to a pool of sera from individuals with high antibody titers to a panel of antigens. The end point titers of 22 Kilifi adults derived from a standard curve of PHIS are in agreement with the findings of Miura *et al.*, and show that the use of a pool of sera from malaria exposed individuals or vaccinated subjects can reliably estimate arbitrary antibody units for samples using a single serum dilution (Miura *et al.* 2008a). This approach has been adopted by several studies to quantify vaccine-induced antibodies (Dicko *et al.* 2008, Malkin *et al.* 2005, Mullen *et al.* 2008, El Sahly *et al.* 2010, Sagara *et al.* 2009) naturally-acquired antibodies (Weiss *et al.* 2010, Miura *et al.* 2008b, Miura *et al.* 2013a) or antibodies to other infectious agents (Seo *et al.* 2013). The amounts determined above were strongly correlated with relative antibody titres estimated from a standard curve of a purified immunoglobulin preparation obtained from malaria exposed Malawian adults. Both approaches of determining the magnitude of an antibody response are superior to presenting raw OD values by i) assigning accurate titers to samples with OD readings that fall outside the linear range of the OD reading and ii) correcting for normal test variations in antibody quantification. The standard curve run at different times will not yield the exact same OD values for every run and the same also applies to the test samples. However, since the concentration of the sample is calculated using the standard curve generated for every run, any variability can be negated. In addition, OD readings obtained in different settings are dependent on the ELISA protocol used and it is therefore unlikely that two different assays will yield the same OD readings.

Standardization of results could be achieved by worldwide distribution of a standard serum, leading to a better ability to compare results between research groups when assessing the magnitude of antigen-specific antibody responses.

A serum dilution of 1:16000 was selected as the optimal dilution to test adult serum samples in this population. At this dilution, the OD₄₉₂ of all samples fell within the linear range of the standard curve hence allowing accurate determination of relative antibody concentrations. In addition, the minimum and maximum detectable concentration of specific-antibodies at this serum dilution was determined for each antigen. Apart from allowing the correct assessment of antibody concentrations with fewer dilutions compared to the end point titer method, testing serum at a 1:16000 dilution is ideal for cohorts with limited volumes of sera such as those obtained from very young children. Inevitably, some individuals had very low antibody titres which could not be quantified at this dilution. These samples were retested at 1:1000 and 1:500 dilutions and those that were not quantifiable at these dilutions were assigned the minimum concentration recorded at the lower limit of detection of the assay.

To obtain reliable data, the standard curve must fulfill several criteria for acceptable quality (Miura et al. 2008a). First, the R-squared value approached unity indicating a good fit of the data to the curve, in all assays. It was also shown that the shape of the dilution curves of all test sera were similar to that of the standard (Figure 3.2). Second, the standard serum pool (PHIS) was obtained from a population similar to the test population. If each sample had a different dilution curve from the standard curve, the antibody units calculated from the OD values would be incorrect, due to the fact that at one dilution the antibody units derived from the standard curve would be different from the units calculated at another dilution (Wernette et al. 2003). Similarly, we've shown that the dilution curve of a pool of IgG obtained from a Malawian adult population (MIG) had a shape similar to that of Kilifi adults tested here suggesting that a standard pool generated from the same species as the test population can be used to reliably determine antibody amounts.

Although the quantification method tested in this chapter yielded reproducible results on different days (Figure 3.8), the robustness of the assay which is defined as the variability of

antibody units with varying experimental conditions such as varying incubation times for antigen coating, serum binding, secondary antibody binding and substrate development was not assessed. Second, a recent study has described a method of converting end point titers into absolute antibody concentrations using a conversion factor (Miura et al. 2009). This approach has been adopted by several animal and human studies (Miura et al. 2009, Mullen et al. 2008, Ellis et al. 2010, Pierce et al. 2010, Sheehy et al. 2011, Sheehy et al. 2012a) but was not explored in this chapter due to the limitation of recombinant antigens to perform affinity-purification of antigen-specific IgG antibodies.

The results in this chapter do not demonstrate superiority of the use of end point titers that can be determined from a single serum dilution (Miura et al. 2008a) over those determined relative to a standard reference reagent prepared from a pool of individuals with high titre antibodies to a panel of antigens of interest. However, the use of a common reference serum with these ELISA quantification methods across study sites is recommended in order to standardize readings obtained on different days, using different ELISA protocols between and within different geographical settings. For other infectious diseases, for instance pneumococcal infection, a standard reference reagent is available to enable harmonization of antibody measurement (Quataert et al. 1995). The use of similar antigens across studies for malaria antigens would also allow for better comparison of findings.

In subsequent chapters of this thesis, I used the malaria immune globulin preparation as the standard reference reagent to titrate the standard curves across assays, assuming an arbitrary antibody concentration of 50AU against all antigens tested. This facilitated direct quantitative comparison of anti-merozoite IgG responses in different cohorts in Kilifi revealing significant differences in antibody amounts in cohorts recruited during varying malaria transmission intensities.

CHAPTER FOUR

A threshold concentration of anti-merozoite antibodies is required for protection from clinical episodes of malaria

4.0 Introduction

Antibodies play an important role in mediating protection against clinical malaria. Purified total IgG obtained from malaria-immune African adults was successfully used to treat children and adults hospitalized with malaria, while control sera from adults not exposed to malaria had no protective effect (Cohen et al. 1961, Sabchareon et al. 1991). Identifying the target(s) of these “protective” antibodies continues to be a priority for malaria vaccine development. Immuno-epidemiological studies are widely used to assess the potential protective efficacy of antibodies against *Plasmodium falciparum* antigens in humans. However, such studies have often yielded inconsistent results with some studies demonstrating a protective role for antibodies to a specific antigen, while others do not (Fowkes et al. 2010). One important reason for this may be the lack of a standardized approach to the reporting of antibody concentrations, and the methods used for their analysis (Fowkes et al. 2010, John et al. 2005). While there is reasonable agreement that high levels of antibodies are better indicators of protection than sero-positivity, the definition of “high” varies considerably between studies (John et al. 2005, Osier et al. 2008, Courtin et al. 2009, Reiling et al. 2010, Richards et al. 2010, Stanisic et al. 2009), making it difficult to compare findings from different sites.

Here, we investigated why antibodies appeared to be protective in some settings but not in others. Specifically, we tested the hypothesis that a threshold concentration of antibody was required for protection and that in some settings, although antibodies were present, their concentrations were below the thresholds required for protection. Quantitative

correlates of protection have been reported for vaccine-induced antibodies against many infectious diseases (Plotkin 2008). For malaria, although antibodies to several specific antigens have been shown to correlate with protection from clinical episodes of malaria (Fowkes et al. 2010), similar quantitative correlates have not yet been defined. In one study, the concept of an antigen-specific threshold concentration of antibodies that correlated best with protection against *P. falciparum* infection was explored (John et al. 2005) but not applied in subsequent studies (John et al. 2008, McCarra et al. 2011). In this chapter, we develop this concept by using data from one cohort to identify “protective thresholds”, defined as the antibody concentrations against specific antigens that best correlate with protection from clinical episodes of malaria. We subsequently tested the validity of these thresholds in an independent cohort.

Our previous studies have shown that antibodies to specific merozoite antigens were associated with protection from clinical episodes of malaria in the Chonyi cohort (Osier et al. 2007, Osier et al. 2008, Polley et al. 2004, Polley et al. 2006). In subsequent studies conducted in the same geographical area along the Kenyan coast, but during a period of moderate transmission, antibodies to the same panel of merozoite antigens were not associated with protection. We used a purified IgG preparation as a reference reagent to quantify the relative amounts of antibodies against specific antigens (as described in Chapter three) and to standardize the measurement of antibody concentrations in both cohorts. Statistical methods were used to determine the relative IgG concentrations against each antigen that best correlated with protection in the Chonyi cohort. We show that antibody concentrations in the moderate transmission cohort were below the thresholds required for protection.

4.1 Methods

4.1.1 Study population

4.1.1.1 Chonyi cohort

The study was undertaken in Chonyi village in Kilifi County on the Kenyan coast. This cohort has been extensively studied (Polley et al. 2006, Polley et al. 2004, Osier et al. 2007, Osier et al. 2008, Agak et al. 2008, Mackintosh et al. 2008a, Mackintosh et al. 2008b, Mwangi et al. 2005, Bejon et al. 2010, Mbogo et al. 2003) and a detailed epidemiological description is published elsewhere (Mbogo et al. 2003, Mwangi et al. 2005). The parasite prevalence rate in children aged 2-10 years (*Pf*PR₂₋₁₀) and the entomological inoculation rate (EIR) were 44% and 10-100 bites/person/year, respectively, at the time of the study. For the current study, we analyzed 286 serum samples collected in October 2000 at the start of a malaria transmission season from children aged 0-10 years. These children were subsequently followed up for 6 months for clinical episodes of malaria by active and passive case detection. In this area, the age-specific criteria for defining clinical episodes of malaria are established and are as follows: for children < 1 year old, a temperature of >37.5°C plus any parasitemia; for children > 1 year old, a temperature of >37.5°C plus a parasitemia of >2500/μl (Mwangi et al. 2005). Trained field workers visited the participants every week whereby children with fever (axillary temperature >37.5°C) had a blood slide taken. Children with a positive test result were treated with antimalarial drugs. In addition, parents were advised to report to a dedicated outpatient clinic at Kilifi District Hospital if their child developed symptoms of disease at any time.

4.1.1.2 Junju cohort

In a second independent cohort, children aged 1-6 years resident in Junju sub-location within the Chonyi area of Kilifi county were originally recruited into a randomized controlled vaccine trial in 2005 (Bejon et al. 2006) and have been followed up as described above, for clinical episodes of malaria (Bejon et al. 2006, Mwangi et al. 2005). Trained field workers living in the same sub-location were available to conduct passive surveillance. Children born into study households are continuously recruited into the cohort and are followed up weekly at home to document clinical episodes of malaria. Peak malaria transmission occurs during the rainy months of May-July and November to December. Blood samples are collected annually during a cross-sectional survey conducted at the beginning of the malaria transmission season in May. The $PfPR_{2-10}$ rate and EIR in Junju were 29% and 21.7 bites/person/year (Midega et al. 2012) respectively, at the time of sampling. Participants in the Junju cohort area live approximately 25km away from those described above in the Chonyi cohort. We analysed 304 serum samples collected in May 2008 from children aged 1-12 years at the start of the malaria transmission season. Data on 6 months of follow up in the subsequent malaria transmission season are presented here.

4.1.2 Recombinant *P. falciparum* merozoite antigens

All antigens are based on *P. falciparum* and include the 19 KDa C-terminal fragment of merozoite surface protein (MSP)-1 of the Wellcome parasite line (Burghaus and Holder 1994), full-length recombinant AMA1 of the HB3 parasite line (Kocken et al. 2002), MSP-2 of the Dd2 parasite line (Taylor et al. 1995) and MSP-3 of the 3D7 allelic type (Polley et al. 2007, Osier et al. 2007). Responses to two fragments of Glutamate-rich protein (GLURP) representing the N-terminal non-repeat region (GLURP-R0) and C-terminal repeat region (GLURP-R2) were also analyzed (Theisen et al. 1995).

4.1.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Serum IgG responses to individual antigens were measured as described in section 2.4.2. Eleven two-fold serial dilutions of a purified malaria immune globulin (MIG) standard were incorporated into the assay to generate a standard ELISA curve. The four-parameter logistic function was used to fit the standard curve in GraphPad Prism version 4.0 (GraphPad software, San Diego, CA). ELISA OD values of test samples were converted into relative antibody concentrations using parameters estimated from the standard curve, assuming the purified IgG preparation contained 50 arbitrary units of antigen-specific antibodies. A pool of sera from Kilifi adults was included on every plate as a positive control. Sera from twenty Europeans served as negative controls to determine cutoff values for seropositivity, defined as mean optical density plus three standard deviations.

Although antibody responses to similar antigens have been measured previously in the Chonyi cohort (Polley et al. 2004, Polley et al. 2006, Osier et al. 2007, Osier et al. 2008), these serum samples were retested to include a common reference reagent (MIG) that allowed standardization and direct comparison of relative antibody concentrations in the two sites. Responses to AMA1, MSP-2 and MSP-3 were measured in both cohorts whereas those to MSP-1₁₉ and GLURP were measured in the Chonyi cohort only.

4.1.4 Statistical analysis

Data analysis was performed using Stata 11 (StatCorp, TX, USA). A modified Poisson regression model was used as previously described (Osier et al. 2008) to examine the effects of individual antibodies on the outcome, defined as a clinical episode of malaria during six months of follow-up. The confounding effects of age and previous malaria exposure were taken into account by fitting age and reactivity to parasite schizont extract as covariates in multivariate analyses. Age was fitted as a categorical variable (age bands

of 0-3, 4-5, 6-7 and 8-12 years) while reactivity to schizont was fitted as a continuous variable.

4.1.4.1 Establishing the presence of a “dose-response” relationship between antibodies and outcome in the Chonyi cohort

First, we evaluated the effect of increasing antibody titers on protection against clinical episodes of malaria. Antibody levels were divided into quartiles (25th, 50th and 75th respectively) based on distribution of antibody concentrations of all individuals in the Chonyi cohort. The 25th, 50th and 75th percentile boundaries were applied as cutoffs for Low, Medium and High antibody amounts, respectively. The risk of disease between individuals who had antibodies below and those above each cutoff point was determined using a generalized linear model.

4.1.4.2 Identifying the antibody concentrations associated with protection

Next, we used data from all children in the Chonyi cohort to estimate the concentration of antibodies that best correlated with clinical protection for each antigen as follows; i) different antibody concentrations were applied as cutoffs for high versus low responders over a range of increasing concentrations up to the maximum concentration recorded against each antigen, ii) a modified Poisson regression model was used to calculate the risk ratio at each cutoff value, iii) the best fitting model was selected using the log pseudolikelihood (Olotu et al. 2011). The antibody concentrations that resulted in the best fitting models were designated as “protective thresholds”. As an independent validation, the protective thresholds defined in the Chonyi cohort were tested in an unrelated cohort (Junju).

4.1.4.3 Comparing analyses based on protective thresholds with those based on conventional cutoffs

The protective thresholds were used in two ways: (i) to compare age-matched antibody levels in the Chonyi versus Junju cohorts and (ii) as cutoffs, comparing the clinical outcome of children with levels above, versus below the threshold, for each antigen in the Junju cohorts.

Next, analyses using the protective threshold as a cutoff point were compared to conventional analyses where the cutoff is defined as 1) seropositivity, defined as the mean plus 3 standard deviations of negative controls, and 2) high versus low antibody levels, defined as the ELISA OD level above which the risk of malaria was lower than the population's average risk of acquiring a clinical episode of malaria (Osier et al. 2008). Importantly, analyses based on high versus low antibody levels have been defined differently in different immuno-epidemiological studies, but ultimately depend on the range of ELISA OD reactivities observed in the population under test.

4.2. Results

4.2.1. Effect of varying antibody concentration and protection against clinical episodes of malaria in the Chonyi cohort.

In order to determine whether there was a “dose-response” relationship between antibodies and the risk of a clinical episode of malaria in the Chonyi cohort, the protective efficacy of antibodies at different cutoff points for Low, Medium and High antibody responses was evaluated (Fig 4.1). When all individuals in the cohort were considered, the risk of disease gradually declined when the cutoff was shifted from Low, Medium to High antibody levels for all antigens tested except MSP-1₁₉, though none of the estimates were statistically significant (RR; 0.74 to 1.83, $p > 0.05$) for all antigens tested.

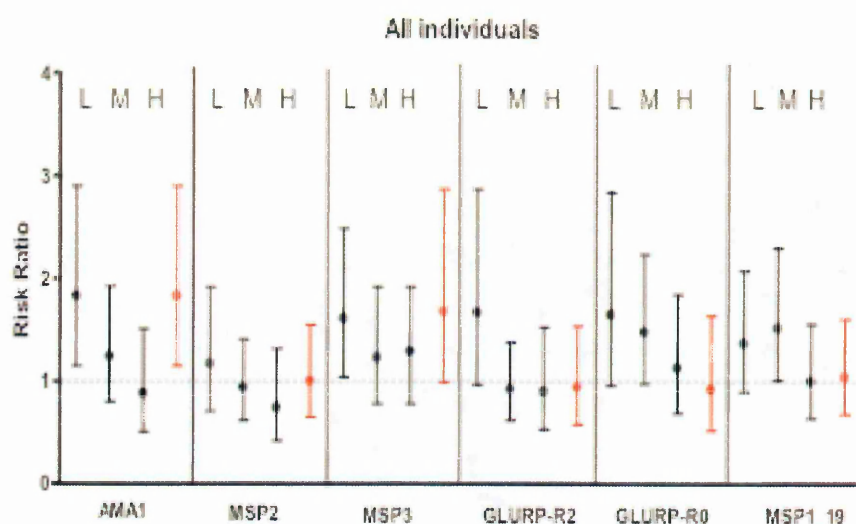


Figure 4.1: Protective efficacy of antibodies based on different cutoff levels. Antibody titers were divided into quartiles (25th, 50th and 75th) based on the distribution of antibody amounts in the Chonyi cohort. Low (L), Medium (M) and High (H) cutoffs were generated based on the 25th, 50th and 75th quartile boundaries, respectively, and applied to all individuals in the cohort. Risk ratio values in red are based on the conventional cutoff of seropositivity defined as Mean+3SD of 20 European sera for each antigen tested.

4.2.2. Protective efficacy of antibodies to merozoite antigens in the Chonyi cohort

The association between antibodies to individual merozoite antigens and the risk of clinical malaria during the follow-up period was analysed using a modified poisson regression model as previously described (Osier et al. 2008). Two ELISA OD cutoffs were tested, seropositivity (defined as mean plus three standard deviations of non-malaria exposed European sera) and high antibody levels (defined as the ELISA OD level above which the risk of disease was lower than the population's average risk of acquiring a clinical episode

of malaria). Among the children who had microscopically detectable parasites at the time of sampling, antibody concentrations above the seropositivity and high antibody level cutoffs were associated with protection against developing a clinical episode of malaria during the follow-up period consistent with previous studies in which ELISA OD values had been used to analyse the data (Osier et al. 2007, Osier et al. 2008, Polley et al. 2006, Polley et al. 2004). Antibody responses to the GLURP-R0 and GLURP-R2 fragments were tested for the first time in this cohort, but neither antibody seropositivity to GLURP-R2 nor GLURP-R0 was associated with protection from malaria, in the whole cohort (RR; 0.94, 95% CI 0.58-1.54; $p=0.83$ and RR; 0.92, 95% CI 0.52-1.64; $p=0.80$, respectively). Following stratification by slide status at the time of sampling, these antibodies were associated with a decreased risk of developing an episode of malaria, in the parasite positive children, although the estimates were not statistically significant in multivariate analyses (RR; 0.59, 95% CI 0.34-1.02; $p=0.06$ and RR 0.56, 95%CI 0.29-1.09; $p=0.09$, for GLURP-R2 and GLURP-R0; respectively). In parasite negative individuals, antibodies to GLURP-R2 and GLURP-R0 were associated with an increased risk of disease (RR; 1.25, 95% CI 0.51-3.08; $p=0.62$ and RR; 1.29, 95% CI 0.50-3.34; $p=0.59$, respectively).

4.2.3. Protective efficacy of antibodies to merozoite antigens in the Junju cohort

Children in the Junju cohort were sampled during a period of moderate malaria transmission intensity. In spite of the fact that malaria transmission intensity was lower in the Junju area than that observed in the Chonyi area during sampling, antigen-specific antibodies were readily detectable in this cohort, and antibody prevalence was 67%, 31% and 26% for AMA1, MSP-2 and MSP-3, respectively. Antibody levels increased significantly with age for all antigens tested (Pearson's chi-square test for trend, $p<0.05$). Analyses based on both seropositivity and high antibody level cutoffs (to classify responders versus non-responders) revealed that antibodies to these antigens were not

associated with protection from malaria, with the exception of MSP-3 (Table 4.1). Unlike the Chonyi cohort where stratification based on slide status was associated with a significant reduction in risk of developing an episode of malaria in the group of children who were parasitemic at the time of sampling, the same was not observed in the Junju cohort.

Table 4.1. A Comparison of Analyses Based on Antibody Seropositivity, High Antibody Levels and Protective Thresholds in the Junju Cohort.

	AMA-1			MSP-2			MSP-3		
	Seropositivity	High antibody levels	Threshold	Seropositivity	High antibody levels	Threshold	Seropositivity	High antibody levels	Threshold
All individuals (N=304)									
Prevalence	67	21	6	31	25	10	26	18	8
% (n/N)	(203/304)	(65/304)	(17/304)	(95/304)	(77/304)	(29/304)	(80/304)	(54/304)	(23/304)
IgG positive^a	42	37	6	37	35	31	30	22	13
	(85/203)	(24/65)	(1/17)	(35/95)	(27/77)	(9/29)	(24/80)	(12/54)	(3/23)
IgG negative^b	40	42	43	43	43	42	45	45	43
	(40/101)	(101/239)	(124/287)	(90/209)	(98/227)	(116/275)	(101/224)	(113/250)	(122/281)
Univariate	1.05	0.87	0.13	0.85	0.81	0.73	0.66	0.49	0.33
RR(95%CI)	(0.79,1.41)	(0.61,1.24)	(0.02,0.91)	(0.62,1.16)	(0.57,1.14)	(0.41,1.28)	(0.46,0.95)	(0.29,0.82)	(0.11,0.95)
Multivariate	1.09	0.94	0.16	0.79	0.78	0.74	0.63	0.47	0.34
RR(95%CI)	(0.75,1.59)	(0.65,1.36)	(0.02,1.12)	(0.57,1.08)	(0.55,1.12)	(0.42,1.29)	(0.42,0.94)	(0.27,0.81)	(0.12,0.94)

Abbreviations: Threshold, protective threshold; ND, not determined; RR, risk ratio.

The risk of developing a clinical episode of malaria during the 6-month follow-up period was compared in analyses based on antibody seropositivity, high antibody levels and protective thresholds. Data were fitted to modified Poisson regression models, adjusting for age and reactivity to parasite schizont extract in multivariate analyses. Significant results at $P < 0.05$ are shown in bold.

^aIgG seropositive, high antibody levels or above the protective threshold and developing malaria during follow-up. ^bIgG seronegative, high antibody levels or below the protective threshold and developing malaria during follow-up. Protective efficacy of thresholds defined in the Chonyi cohort^c versus those defined in the Junju cohort^d using the log maximum likelihood method.

4.2.4 Identifying a threshold concentration of antibodies that best correlates with a reduced risk of malaria

Next, we tested the hypothesis that the apparent lack of protection observed in the Junju cohort could be explained by insufficient antibody concentrations. To do this, we used data from all children in the Chonyi cohort where antibodies to these antigens were shown to correlate with protection from malaria. Using the purified IgG as a standard for measuring relative antibody concentrations, we determined for each antigen the relative IgG antibody concentration that best correlated with protection from malaria by selecting the model with the least log pseudolikelihood (Olotu et al. 2011). The antibody concentrations that resulted in the best fitting models were designated as “protective thresholds” and it varied for different antigens, resulting in protective efficacies $((1-RR) \times 100)$ of 25-56% (Table 4.2). Notably, the threshold concentration was higher than that defined both by seropositivity and by high antibody levels. Relative antibody amounts against the R0 fragment of GLURP were highly skewed to the right and it was not possible to define high levels as described above. A protective threshold could not be identified for antibodies against MSP-1₁₉.

Table 4.2. Cutoff Values for Antibody Seropositivity, High Antibody Levels and Protective Thresholds in the Chonyi Cohort.

Antigen	Seropos cutoff ^a	High levels cutoff ^b	Protective threshold ^c	Protective efficacy ^d (%)	(Risk Ratio; 95% CI) ^e
AMA-1	0.70	43.70	55.00	25	(0.75; 0.42-1.33)
MSP-2	7.40	18.50	19.00	43	(0.57; 0.32-1.00)
MSP-3	0.39	6.50	16.00	56	(0.44; 0.16-1.17)
GLURP-R0	7.90	ND	11.00	41	(0.59; 0.27-1.28)
GLURP-R2	4.30	5.90	8.00	40	(0.60; 0.31-1.18)
MSP-1 ₁₉	8.2	15.70	ND	ND	ND

Abbreviations: AU, arbitrary units; SD, standard deviation, ND, Not Determined.

^aSeropositivity cutoff is defined as the mean + 3SD response of sera from malaria naïve donors for each antigen tested.

^bHigh antibody level cutoff is defined as the antibody concentration above which the risk of malaria was lower than the population’s average risk of developing malaria.

^cProtective threshold cutoff is defined as the antibody concentration that resulted in the least log pseudolikelihood value in a modified Poisson regression model.

^dProtective efficacy in analyses based on the protective threshold as a cutoff. Calculated as (1-RR)*100.

^eRisk ratio estimates employed to calculate protective efficacy.

4.2.5. Antibody concentrations are below protective thresholds in Junju cohort.

We then compared antibody concentrations in age-matched children from Chonyi and Junju in relation to the protective thresholds for each antigen. Median IgG levels to AMA1, MSP-2 and MSP-3 were significantly lower in the Junju cohort compared to those in the Chonyi cohort across all age groups (Mann-Whitney test $p < 0.01$) except the youngest children (0-3 years), who had similar median antibody levels to AMA1 in both cohorts (Figure 4.2). Importantly, the proportion of age-matched children that had antibody concentrations above the protective thresholds was also significantly lower in Junju compared to Chonyi for AMA1 and MSP-2 except the youngest age-group (Fisher's exact test $p < 0.01$). The proportion of children that had protective threshold antibody concentrations against MSP-3 was significantly lower in Junju compared to Chonyi only in the 4-5 year age group.

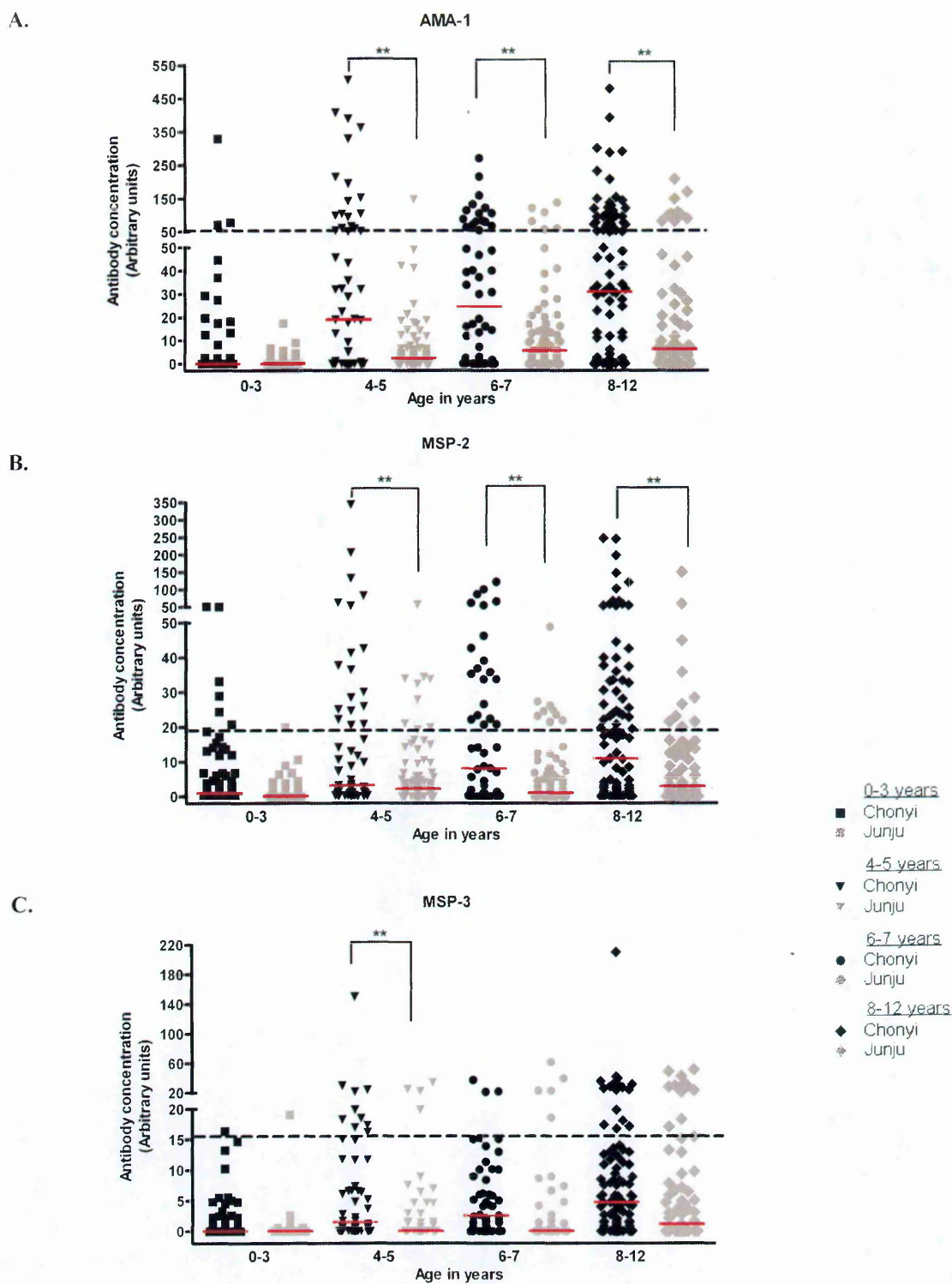


Figure 4.2: Distribution of antibody concentrations in age-matched children recruited from the Chonyi and Junju (grey) cohorts against (A) AMA1, (B) MSP-2 and (C) MSP-3. Black dotted and red bold lines represent the protective threshold levels and the median antibody concentrations by age against each antigen, respectively.

4.2.6. Protective thresholds explain apparent lack of protection in the Junju cohort

We used the protective thresholds derived in the Chonyi cohort to classify children in the Junju cohort as having antibodies above or below threshold concentrations for each antigen. We then used the modified Poisson regression model as previously described, to compare the outcome in Junju children classified in this manner. We compared these results with those obtained from analyses based on seropositive versus seronegative, and high versus low levels. For antibodies against AMA1 and MSP-3, we found that the estimates of risk decreased in a stepwise fashion, when the cutoff was applied as seropositivity, high levels, and finally as protective thresholds defined independently for both Chonyi and Junju. For example, using these three cutoff points in multivariate analyses, antibodies against AMA1 were associated with relative risks (95% confidence intervals) of 1.09(0.75-1.59), 0.94(0.65-1.36) and 0.16(0.02-1.12), respectively. For both AMA1 and MSP-3, antibody concentrations at the protective thresholds were considerably higher than those at seropositive or high levels (Table 4.1). Of note, the proportion of children with antibody concentrations above any given cutoff reduced considerably as the cutoff was raised from sero-positivity, to that defined by high versus low levels, through to protective thresholds. In the Junju cohort overall, less than 10% of children had antibodies above defined threshold levels either in the Chonyi cohort (5.6%, 9.5%, and 6.9%) or Junju cohort (6%, 5.5% and 5%) for AMA1, MSP-2 and MSP-3, respectively.

4.3 Discussion

An increasing body of evidence suggests that protection from malaria is dependent on high antibody concentrations (John et al. 2005, Osier et al. 2008, Courtin et al. 2009, Reiling et al. 2010, Richards et al. 2010, Stanisic et al. 2009). My data build on this by using a standardized reference reagent to quantify relative antibody concentrations and to define the threshold concentration for each antigen that was associated with protection against clinical episodes of malaria, using data from a high malaria transmission cohort. Application of these thresholds to an independent moderate transmission cohort provided an explanation for the lack of protection observed in the latter cohort. Antibody levels in age-matched children were significantly lower in the moderate (Junju) compared to those observed in the high (Chonyi) transmission cohort. Consequently, only a small proportion of children in Junju achieved antibody levels above the protective threshold concentrations. These data thus provide a plausible biological explanation for the observation that antibodies to individual antigens are associated with protection in some cohorts, but not others (Fowkes et al. 2010).

Methodological differences between immuno-epidemiological studies make it difficult to interpret apparently contradictory results where antibodies to a single antigen are associated with protection from malaria in one geographical setting, but not in another (Fowkes et al. 2010). These differences range from definition of end-points, whether clinical malaria, time to infection, or malaria with high parasitaemia, for example, to duration of follow up, quality and allelic type of antigen tested, whether or not full-length or fragments of antigens were tested, right through to laboratory assays and analytical approaches, among others. In the current study, we minimized all these methodological differences and conducted the studies identically.

There was a clear difference in parasite prevalence rates in children aged 2-10 years ($PfPR_{2-10}$) (Noor et al. 2009) in the Junju cohort (29%) as compared to Chonyi (44%).

Several studies have shown that parasite prevalence rates in children aged 2-10 years are reliable indicators of malaria endemicity (Noor et al. 2014, Noor et al. 2009, Smith et al. 2007, Smith and Hay 2009, Hay, Smith and Snow 2008). From these studies, areas of high transmission are defined by a $PfPR_{2-10}$ of $\geq 40\%$ whereas those of low transmission are defined by a $PfPR_{2-10}$ of $< 5\%$. Areas of intermediate/moderate transmission which would experience an immediate reduction in parasite prevalence following large-scale deployment of insecticide-treated nets have a $PfPR_{2-10}$ of 5 - 40% (Hay et al. 2008, Smith and Hay 2009).

Apart from differences in malaria transmission intensity, the two cohorts belong to the same ethnic group and share similar environmental factors such as cultivation practices, rainfall, wind direction, presence of streams and rivers. However, notable differences between both cohorts were the change in antimalarial drug policy from sulphadoxine-pyrimethamine to artemether lumefantrine in 2006 (Amin et al. 2007) and the distribution of free insecticide-treated bednets by the government in the same year (Noor et al. 2007) increasing coverage from 6% in 1999 (Mwangi et al. 2005) to more than 60% (Noor et al. 2007). It is plausible that these factors also contributed to the decline in malaria transmission. In these circumstances, and using a malaria IgG reference serum to standardize antibody measurements across both cohorts, we were able to show antibody concentrations in the Junju cohort were significantly lower than those in Chonyi.

Immuno-epidemiological studies have traditionally classified study participants as being seropositive or seronegative for responses to specific antigens. More recently, we and others have found that classifying individuals as having high or low levels of antibodies is a highly informative indicator of protection among children (John et al. 2005, Osier et al. 2008, Courtin et al. 2009, Reiling et al. 2010, Richards et al. 2010, Stanisic et al. 2009). However, the actual definition of high versus low antibody levels varies between studies, making it difficult to meaningfully compare data from different sites. We had previously

derived a definition for high versus low antibody levels that was based on the range of responses observed in the cohort under study (Osier et al. 2008). By this definition, we found that antibody levels which we would have considered to be high in Junju cohort, were nevertheless lower than those required for protection in the Chonyi cohort. Thus, an analysis based on high versus low levels in the Junju cohort would have erroneously concluded that high levels of antibodies against these merozoite antigens were not associated with protection. I therefore propose an analytical approach based on the principle of “protective thresholds” in place of (or in addition to) seropositivity, or varying definitions of high versus low antibody levels. The protective threshold concentrations for antibodies against each antigen are fixed and the confidence intervals around these estimates will not vary from one population to the next, allowing for efficient comparison of data across sites. Although the estimates of risk in the Junju cohort did not always reach statistical significance, I observed a clear trend in the reduction of risk when the analysis was based on protective thresholds. In effect, this was a cutoff higher than that conventionally used for seropositivity and/or high versus low levels of antibodies. An obvious consequence of raising cutoff points is a reduction in the ability to detect significant effects, as the numbers achieving higher antibody concentrations may be relatively small, particularly in areas of low malaria endemicity. The protective threshold concentration I derived varied by antigen. For some antigens, for example AMA1 and MSP-3, this concentration was considerably higher than that defined by sero-positivity or high levels. For MSP-2, the protective threshold concentration was substantially higher than that defined by seropositivity, but was nearly equivalent to what I had previously defined as high levels (high antibody level cutoff was 18.5 AU whereas the protective threshold cutoff was 19 AU). For antibodies against GLURP, the difference between the protective threshold concentration and that defined by sero-positivity, and/or high levels (GLURP-R2) was less marked. For MSP-1₁₉, I was not able to define a protective

threshold by the methods presented here. These findings need to be validated in much larger cohorts, and in samples collected from different geographical settings.

Additionally several studies have shown that quality of antibody responses, in particular the type of IgG sub-classes are also important for protection (Courtin et al. 2009, Stanisic et al. 2009). I did not set out to study IgG subclasses in the current study. However, antibodies against the antigens I have analyzed here have been shown from multiple studies to comprise predominantly of the cytophilic IgG1 and IgG3 subclasses (Metzger et al. 2003, Polley et al. 2004, Osier et al. 2007) even in areas of low malaria transmission intensity (Scopel et al. 2006). Thus although useful, measurement of IgG subclasses in this study would not have altered my interpretation of the data. In the present study, I measured responses to a single allelic variant of each antigen and did not ascertain whether the circulating parasite strains were bearing the haplotypes tested or whether the strains were similar in the two cohorts. Although this may be a potential limitation, our previous studies conducted in the same geographical area at different time points have found a high correlation between antibodies against different allelic versions of AMA1 (Osier et al. 2008, Osier et al. 2010b), MSP-2 (Osier et al. 2008, Osier et al. 2010a) and MSP-3 (Osier et al. 2008, Osier et al. 2007). Furthermore, a longitudinal study conducted in The Gambia showed that circulating alleles remained stable over time (Conway, Greenwood and McBride 1992).

In conclusion, my data suggest that a “protective threshold” concentration of antibodies against specific merozoite antigens of *P. falciparum* needs to be achieved for protection from clinical episodes of malaria. I propose a new approach to the analysis of such data that may add value to current analytical strategies. If validated in larger studies and in unrelated immuno-epidemiological cohorts, this analytical approach based on “protective thresholds” could be usefully extended to the testing of immunogenicity and potential protective efficacy of sub-unit vaccines currently under development for malaria.

CHAPTER FOUR (SUPPLEMENT)

Exploratory post-hoc analysis

A comparison of threshold antibody concentrations associated with protection against clinical episodes of malaria in two villages in Kilifi and in Tanzania

In the previous section of this chapter, I have demonstrated that children in the moderate transmission cohort (Junju) who achieved antibody titres above the protective thresholds defined in the high transmission cohort (Chonyi) had a reduced risk of developing a clinical episode of malaria during the follow-up period. However, the protective threshold values were derived as point estimates, with no estimation of the level of uncertainty around these cutoff values. Here, I sought to obtain the confidence intervals around the threshold estimates using the bootstrap percentile method (Efron, 1993, Hollander, 2004) based on at least 1000 bootstrap samples.

Additionally, the thresholds identified in the previous section were based on a specific primary dataset with validation of these cutoffs in an independent cohort. Here, I also sought to determine whether similar threshold estimates could be obtained from an independent dataset to provide further evidence in favor of a threshold effect. I therefore compared the optimal cut offs generated independently in the high and low transmission cohorts (Chonyi and Junju) using similar methodologies.

Methods

In the Chonyi cohort, antibody titres were fitted into a modified poisson regression model and a dichotomization point that maximized the log likelihood that antibody titres would predict protection from malaria was selected as the optimal fit. Bootstrapping was used to calculate the 95% confidence intervals around the selected cutoff.

The Chonyi cohort was used as the primary cohort to build the models. Similar models were applied to the Junju dataset to compare and confirm the validity of the protective thresholds obtained in the Chonyi cohort.

Results

The confidence intervals around the protective thresholds previously defined in the Chonyi cohort were as follows: AMA1 55AU (37-63AU), MSP-2 19AU (9-26AU), MSP-3 16AU (12-29AU), GLURP-R0 11AU (6-22AU) and GLURP-R2 8AU (4-15AU) (Figure 4.3). Protective thresholds for MSP-1₁₉ could not be estimated as the dichotomization point that maximized the log likelihood that this cutoff would predict protection corresponded to the highest MSP-1₁₉ antibody titre (Figure 4.3 F).

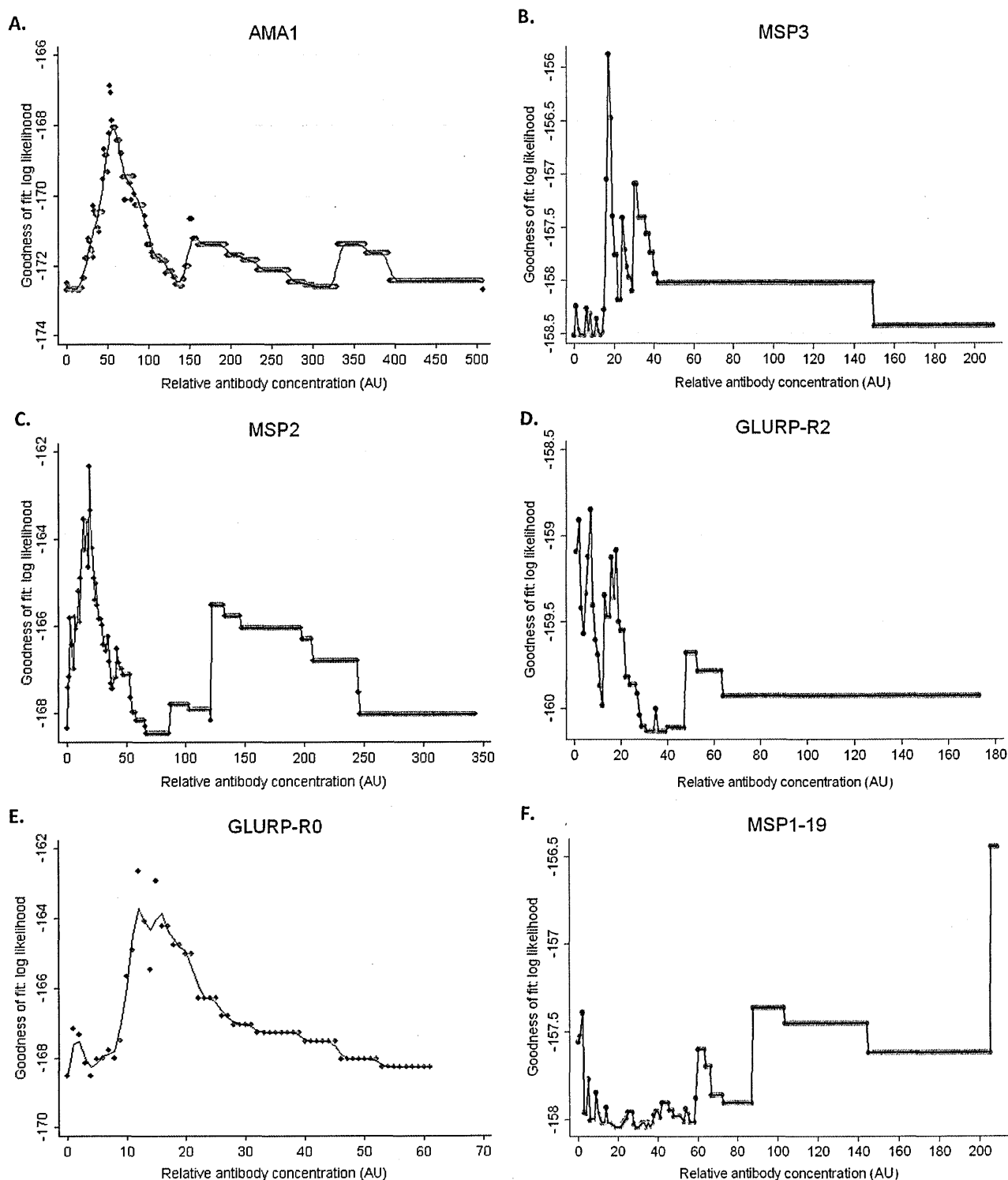


Figure 4.3 Plots of goodness of fit log likelihood estimates and the dichotomization point for the range of antibody titres obtained for (A) AMA1 (B) MSP-3 (C) MSP-2 (D) GLURP-R2 (E) GLURP-R0 and (F) MSP-1₉ in the Chonyi cohort. The dichotomization point was selected based on the modified poisson regression model that gave the least log likelihood (goodness of fit) estimate. The 95% confidence intervals of the protective thresholds were estimated by a bootstrap method.

Further validation of the protective threshold estimates obtained in the high transmission Chonyi cohort was performed by independently running similar models in the moderate transmission Junju cohort (Figure 4.4). Interestingly, the protective threshold cutoffs and 95% confidence intervals defined in the Junju cohort were consistent with those defined in the Chonyi cohort (50-57AU (CI 39-64) versus 55AU (CI 37-63) for AMA1; 20AU (CI 9-22) versus 16AU (12-29) for MSP-3; and 24AU (CI 13-31) versus 19AU (CI 9-26) for MSP-2), respectively (Table 4.3).

In a recent study conducted in Tanzania, protective threshold levels to AMA1 (3D7), AMA1 (FVO), MSP-1₁₉, MSP-2 (CH150), MSP-2 (Dd2), MSP-3 (3D7) and MSP-3 (K1) were defined using a similar methodological and analytical approach (Rono et al. 2013). In their study, Rono *et al.* defined the protective levels for MSP-2 (Dd2) and MSP-3 (3D7) as 11AU and 14AU, respectively. These estimates were comparable to those defined in the Chonyi and Junju cohorts (19AU and 24AU for MSP-2) and (16AU and 20AU for MSP-3, respectively) hence strengthening the suggestion that these threshold concentrations could be biologically relevant and could be applied as reliable correlates of protection against development of clinical episodes of malaria.

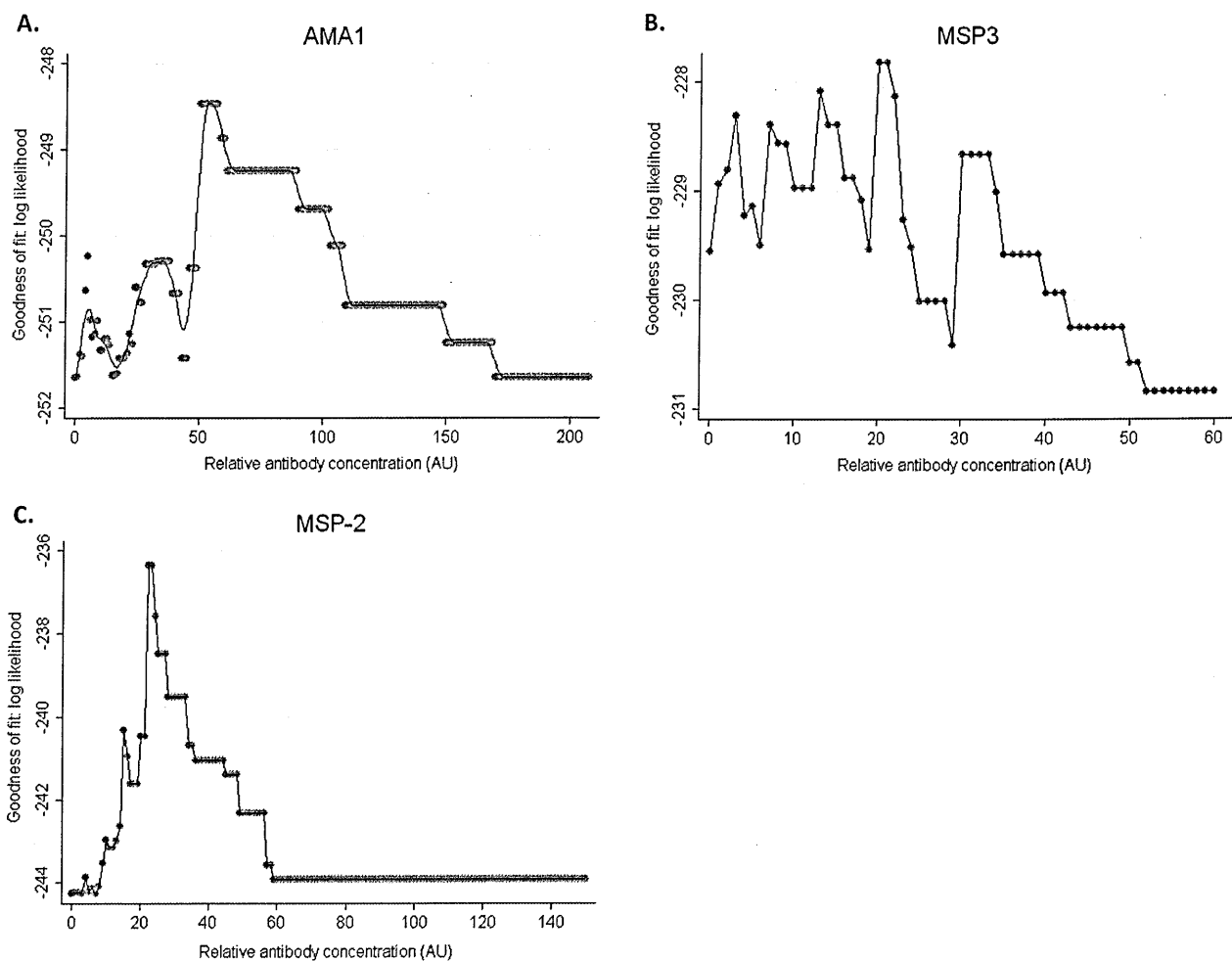


Figure 4.4 Plots of goodness of fit log likelihood estimates and the dichotomization point for the range of antibody titres obtained for (A) AMA1, (B) MSP-3, and (C) MSP-2 in the moderate transmission Junju cohort. The dichotomization point was selected based on the modified poisson regression model that gave the least log likelihood (goodness of fit) estimate. The 95% confidence intervals of the protective thresholds were estimated using a bootstrap method.

Table 4.3. Protective threshold concentrations and the 95% confidence interval estimates derived independently in the Chonyi, Junju and Tanzanian cohorts.

	Chonyi cohort	Junju cohort	Tanzania cohort
	Threshold (95% CI)	Threshold (95% CI)	Threshold ¹
AMA1(HB3)	55 (37-63)	50 – 57(39-64)	ND*
MSP-3(3D7)	16 (12-29)	24 (13-31)	14
MSP-2(Dd2)	19 (9-26)	20 (9-22)	11
GLURP-R0	11 (6-22)	ND*	ND*
GLURP-R2	8 (4-15)	ND*	ND*
MSP-1 ₁₉	ND**	ND*	59

¹95% confidence intervals for these threshold estimates have not been assessed (Rono et al. 2013).

ND Not Determined.

ND* IgG responses to these specific antigens were not measured.

ND** I could not define a threshold concentration against MSP-1₁₉ in the Chonyi cohort because the dichotomization point that gave the least log likelihood ratio (best fit) corresponded to the maximum antibody concentration measured in this cohort.

Finally the thresholds defined for Chonyi, Junju and Tanzanian cohorts were applied in the respective cohorts to determine their association with subsequent risk of developing a clinical episode of malaria. For all antigens tested, the different thresholds defined in the three populations were associated with a reduced risk of developing malaria during the follow-up period (Table 4.4). The reduction in risk of developing a clinical episode of malaria was significant in the three cohorts for individuals who had anti-MSP (Dd2) antibodies above the thresholds defined; (RR 0.57 (0.32-1.00), $p=0.051$; RR 0.26 (0.07-0.93), $p=0.036$; and RR 0.30 (0.15-0.60), for the Chonyi, Junju and Tanzanian cohorts (Rono et al. 2013), respectively). Similarly, antibodies above the threshold cutoff for MSP-3 were associated with a significant reduction in risk of developing malaria in the Junju and Tanzanian cohorts (RR 0.15 (0.02-1.00), $p=0.05$ and RR 0.46 (0.24-0.90), respectively) and towards reduced risk in the Chonyi cohort (RR 0.44 (0.16-1.17).

Table 4.4 A comparison of the protective efficacy of cutoffs defined and applied independently in three different settings (Chonyi, Junju and Tanzania)

	Thresholds (Chonyi) ^a		Thresholds (Junju) ^b		Thresholds (Tanzania) ^c	
	Univariate analysis	Multivariate analysis ^d	Univariate analysis	Multivariate analysis	Univariate analysis	Multivariate analysis
AMAI (HB3)	0.77 (0.45,1.32)	0.75 (0.42,1.33)	0.13 (0.02-0.91)	0.14 (0.02-1.03)	ND ^e	ND
MSP-2 (Dd2)	0.56 (0.32,0.97)	0.57 (0.32,1.00)	0.27 (0.07-1.00)	0.26 (0.07-0.93)	0.24 (0.12-0.46)	0.30 (0.15-0.60)
MSP-3 (3D7)	0.49 (0.19,1.27)	0.44 (0.16,1.17)	0.14 (0.02-0.97)	0.15 (0.02-1.00)	0.41 (0.23-0.72)	0.52 (0.30-0.89)

^aThresholds defined in the Chonyi cohort were 55AU, 19AU and 16AU for AMA1, MSP-2 and MSP-3, respectively.

^bThresholds defined in the Junju cohort were 50-57AU, 24AU and 20AU for AMA1, MSP-2 and MSP-3, respectively.

^cThresholds defined in the Tanzania cohort were 11AU and 14AU for MSP-2 and MSP-3 (Rono et al. 2013).

^dIn multivariate analysis, age and previous exposure to infection (reactivity to schizont extract) were taken into account as potential confounders.

^eND- Not defined. Responses to AMA1 (HB3) allele were not tested in this study (Rono et al. 2013).

Discussion

In the previous chapter, I demonstrated that the apparent lack of protection associated with antibodies against a panel of merozoite antigens in a moderate transmission cohort could be explained by failure to achieve titres above certain protective thresholds. The thresholds were defined in a high transmission cohort (Chonyi) and subsequently validated in the moderate transmission cohort (Junju). Here, I extend the above findings by deriving the protective thresholds independently in each of the two cohorts and comparing these thresholds with those described in a third cohort from a geographically distinct population in Tanzania (Rono et al. 2013). I found that the protective thresholds vary only slightly depending on the population they are derived from. In addition, there was an overlap of the confidence intervals around each threshold estimate when derived independently in each population. For instance, for MSP-2, the Chonyi cohort threshold cutoff was 19AU (95% CI 9-26) and 20AU (95% CI 9-22) for the Junju cohort. The similarities in thresholds and confidence intervals suggest that these estimates are a realistic reflection of the levels of antibodies that are associated with protection against clinical episodes of malaria.

Individuals who had antibodies above the protective threshold concentrations for AMA-1, MSP-2 and MSP-3 defined independently in the moderate transmission Junju cohort had a reduced risk of developing a clinical episode of malaria. Similarly, when those defined in the Chonyi cohort were applied to the Junju cohort, the relative risk ratio was also comparable (Murungi et al. 2013), a finding which may be explained by the similarity in threshold estimates and overlapping confidence limits.

The thresholds defined in the Chonyi and Junju cohorts were consistent with what was observed in a geographically distinct cohort of Tanzanian children (Rono et al. 2013), although the age range studied was slightly wider (1-16 years) compared to the Junju and Chonyi cohorts (1-12 years). The parasite prevalence rate in the Tanzanian cohort was

comparable to the Chonyi cohort ($PfPR_{2-10}$ of 49% versus 44%, respectively), hence classifying both as high transmission cohorts, whereas the Junju cohort with a $PfPR_{2-10}$ of 29% was classified as a moderate transmission cohort (Noor et al. 2014, Smith et al. 2007, Hay et al. 2008). Differences in transmission intensities notwithstanding, the protective thresholds were comparable in the three cohorts studied here and also notably higher than the seropositivity and “high” antibody level cutoffs employed by previous studies (Osier et al. 2008, Osier et al. 2007, Polley et al. 2004, Polley et al. 2006). The similarity in protective threshold cutoffs derived independently in three different settings suggests that these concentrations may be biologically relevant. This study offers for the first time, a range of plausible concentrations that may be useful cutpoints for comparing findings between studies to minimize the inconsistencies in assessing the protective efficacy of antibodies to merozoite antigens.

The two-level model approach used in this chapter to infer threshold cutoffs has the disadvantage of assuming an immediate change in the risk of developing disease at a particular titre, i.e. characterizing individuals close to but on opposite sides of the cutpoint as having very different rather than very similar outcomes, which might be an oversimplification. I did not test how abrupt the change in risk was around the threshold estimates defined here, as has been previously proposed (Olotu et al. 2011). Nonetheless, a linear model that assumes a continuous reduction in risk of developing a clinical episode of malaria with increase in antibody titres did not fit the data better than the traditionally employed seropositivity cutoffs or the two-level model applied here (RR 0.98- 0.99) $P>0.05$ (for most antigens tested).

I cannot rule out the fact that the relatively small sample sizes and low proportion of children achieving titres above the thresholds in the Chonyi and Junju cohorts contributed towards obtaining relatively wide confidence intervals around the defined protective thresholds. One

approach towards resolving this would be testing in a larger dataset. Such a dataset would be adequately powered and would give more confidence in the results. It also remains to be determined whether achieving concentrations above the thresholds correlates with functional activity such as the inability to inhibit parasite growth *in vitro* or effectively bind to other cells such as neutrophils and monocytes to promote parasite clearance by release of soluble mediators or by phagocytosis.

Thus far, there is no immunological measurement that provides a reliable and reproducible measure of clinical protection in different endemic populations and this study has extended our knowledge by offering a range of plausible cutoffs associated with a reduced risk of developing uncomplicated malaria that may be applied in other geographical regions.

CHAPTER FIVE

Antibodies against merozoite antigens and protection against severe malaria in a longitudinally monitored birth cohort of Kenyan children

5.0 Introduction

In malaria endemic areas, the outcome of a *P. falciparum* infection ranges between asymptomatic infections to mild or severe and life-threatening malaria. The latter is particularly observed in children aged less than 5 years, although immunity to this severe form of disease is achieved early in life. Thereafter, these children remain susceptible to episodes of non-severe malaria until early adulthood but continue to encounter asymptomatic infections to which immunity is non-sterilizing (Marsh and Kinyanjui 2006, Langhorne et al. 2008). Evidence from modeling studies suggests that immunity to severe, non-cerebral malaria occurs after one or two episodes of disease (Gupta et al. 1999). The timing and sequential acquisition of immunity first to severe malaria, then to non-severe and later to asymptomatic infections implies that the targets and nature of the immune response against these different manifestations of an infection may be distinct with limited overlap. However, immune responses that act to clear parasites (anti-parasitic immunity) will also reduce the risk of clinical disease. It is unclear whether the same targets and immune responses are equally important for the different outcomes of a malaria infection.

A protective role of antibodies against malaria has been directly demonstrated through the transfer of purified gammaglobulin from immune African adults to Gambian (Cohen et al. 1961 1963) and Thai (Sabchareon et al. 1991) children during an acute episode of malaria leading to a substantial reduction in parasite density and clinical symptoms. In addition, cord

blood obtained from mothers during delivery was successfully used to treat children with malaria (Edozien, Gilles and Udeozo 1962). Recent studies conducted in malaria endemic populations have also demonstrated a role for pre-existing anti-merozoite antibodies (reviewed in (Fowkes et al. 2010)) and antibodies to the infected red blood cell surface (Chan, Fowkes, and Beeson 2014) in protection against clinical episodes of malaria. These studies have largely focused on the non-severe form of malaria as the primary outcome and have also relied on antibody measurements taken at a single time-point prior to the disease episode. Although useful, the latter may not adequately reflect an individual's ability to mount an antibody response, as such responses are largely driven by factors which vary over time such as concurrent parasitemia, number of previous infections and age (Kinyanjui et al. 2009). Studies in humans to determine the protective mechanisms underlying immunity to severe malaria have been largely conducted at the time of acute infection (reviewed in the Introduction). Although such studies are well suited for studying the quality of immune responses induced by parasites during the acute phase of the disease, it is difficult to establish a causal relationship between the particular immune response and protection from severe disease. As such, prospective studies are ideal but have so far been few (Bull et al. 1998, Bull et al. 2002, Ndungu et al. 2002, Osier et al. 2008), limited in sample size (Ndungu et al. 2002, Bull et al. 2002), used inadequate definitions of severe malaria (Bull et al. 1998, Osier et al. 2008) and largely focused on antibody titres measured at a single time-point (Bull et al. 1998, Bull et al. 2002, Ndungu et al. 2002, Osier et al. 2008).

A key and as yet unsolved question is whether pre-existing antibody responses (measured at multiple time-points) play a role in acquisition of immunity to severe malaria and if so, what the specific target antigens are.

5.1 Objectives

To use a case-control approach nested within a longitudinally monitored birth cohort of children aged between 0 – 2 years to:-

- Describe the factors that influence antibody titres against a panel of *P. falciparum* merozoite antigens in infants.
- Describe and compare the longitudinal dynamics of anti-merozoite antibodies from birth until the incidence of severe malaria for cases and their corresponding controls.
- Compare the protective efficacy of anti-merozoite antibodies measured at a single time-point with those measured at several time-points prior to an episode of severe malaria.

5.2 Methods

5.2.1 Recombinant antigens and Multiplex ELISAs.

Antibody levels to a panel of recombinant merozoite antigens; AMA1 (FVO and 3D7 allelic types), MSP-2 (Dd2 and CH150/9 allelic types), MSP-3 (3D7 and K1 allelic types), *Pf*Rh2 and MSP-1₁₉ were measured using a multiplex bead-based ELISA platform, as previously described in Chapter two. IgG responses to schizont extract were measured using a standard ELISA technique as previously described (Ndungu et al. 2002)

5.2.2 Serum and red blood cell pellet samples

Serum and red blood cell pellet samples collected consecutively at three-monthly intervals from birth until the occurrence of a severe malaria episode (for the cases) were assayed and

compared with those collected from matched control subjects who did not develop an episode of severe malaria.

5.2.3 Detection of *P. falciparum* infections by microscopy and PCR

Thin blood smears were prepared from finger prick blood samples, stained with giemsa and examined under a light microscope. Parasites were enumerated against 500 uninfected RBCs or 200 leukocytes. Blood films were independently scored by two expert microscopists. Parasite DNA was extracted from the red blood cell pellets followed by genotyping at the MSP-2 gene locus using a capillary electrophoresis method (Lundblom et al. 2013, Liljander et al. 2009).

5.2.4 Statistical analysis

Continuous variables were compared using the Kruskal-Wallis and Mann-Whitney U rank sum tests. Proportions were compared using the Chi-squared test. The association between antibody titres and age, year of birth, location, concurrent parasitemia and previous exposure to infection (measured by reactivity to schizont extract) were analysed using generalized estimating equation models in a univariate analysis. This model accounts for clustering as a result of multiple antibody measurements obtained per child using an exchangeable correlation structure. Repeated observations on the same subject are not independent so a correction needs to be made for this within-subject correlation. To test whether antibodies were associated with protection from severe malaria, a conditional logistic regression model was applied that takes into account individual matching of cases to controls (upto 3 controls per case).

5.3 Results

5.3.1 Factors that influence antibody titres in children aged 0-2 years

The effect of age, the presence of detectable parasites during the three-monthly sampling points, cumulative exposure as measured by reactivity to schizont extract, year of sample collection and the residence of subjects on the specific antibody titres are summarized in Table 5.1. The coefficients indicate that for every unit increase in the predictor variable the response variable changes by its respective regression coefficient in the log-odds scale. Results of only one allelic type are shown since antibody titres between the two alleles of AMA1, MSP-2 and MSP-3 tested were highly correlated. Increasing age was associated with a significant decrease in antibody titres against all antigens tested (range of coefficients, -3.7 to -17.3, $p < 0.005$). This reflects the decay of maternally transferred antibodies with increasing age. For all antigens tested, the presence of parasites detected either by PCR or microscopy at any of the three-monthly visit samples was associated with a significant increase in antibody titres (range of coefficients, 0.03 to 0.12; $p < 0.005$). Similarly, increasing antibody titres to schizont extract were also associated with a significant increase in antibody titres to the antigens tested ($p < 0.05$). Recruitment into the birth cohort and subsequent follow-ups were conducted between 2001 and 2008, a period of declining malaria transmission in Kilifi county (O'Meara et al. 2010, Okiro et al. 2007). We determined the influence of year of falling malaria transmission on antibody titres by comparing responses from samples collected between 2001 and 2008. Periods of low malaria transmission were associated with an overall decrease in antibody titres for all antigens tested. For instance, for AMA1, a significant stepwise decrease in antibody titres with increasing year was observed over the period of recruitment and sample collection (coefficients of -3.19, -3.75, -6.15, -6.59 and -7.41 in 2003, 2004, 2005, 2006 and 2007 respectively, compared to the first year of recruitment and sample collection - 2002).

Table 5.1. The influence of different factors on the IgG response to a panel of merozoite antigens measured at three-monthly sampling time points from birth upto two years of age.

Factors that influence antibody titres	AMA1(3D7)	MSP2(Dd2)	MSP3(3D7)	PfRh2	MSP-1 ₁₉
Age in months	-17.3(-19.1, -15.5), <0.001	-9.6(-12.4, -6.8), <0.001	-9.1(-10.2, -7.9), <0.001	-3.7(-6.2, -1.2), 0.003	-12.3(-16.9, -7.7), <0.001
Infection status*	0.12(0.10, 0.15), <0.001	0.08(0.05, 0.10), <0.001	0.07(0.05, 0.09), <0.001	0.03(0.01, 0.05), 0.002	0.10(0.06, 0.14), <0.001
Schizont extract response	0.50(0.29, 0.71), <0.001	0.74(0.13, 1.36), 0.02	0.34(0.19, 0.48), <0.001	0.13(0.03, 0.24), 0.007	1.06(0.60, 1.52), <0.001
Year of sample collection					
2002	Ref	Ref	Ref	Ref	Ref
2003	-3.19(-5.88, -0.51), 0.020	0.28(-4.42, 5.00),0.90	-1.33(-2.70, 0.02), 0.05	0.47(-1.56, 2.51), 0.46	2.03(-3.13, 7.21), 0.44
2004	-3.75(-6.32, -1.19), 0.004	-3.13(-5.84, -0.41),0.024	-0.43(-2.41, 1.55), 0.67	-0.08(-1.80, 1.63), 0.92	0.81(-2.34, 3.98), 0.61
2005	-6.15(-8.40, -3.90), <0.001	-4.60(-7.12, -2.09),<0.001	-2.98(-4.09,-1.87),<0.001	-0.23(-1.86, 1.38), 0.77	-0.64(-3.28, 2.00), 0.63
2006	-6.59(-8.87, -4.32), <0.001	-3.46(-6.71, -0.22),0.036	-2.89(-4.11,-1.67),<0.001	0.27(-1.65, 2.19), 0.28	-0.48(-2.90, 1.94), 0.69
2007	-7.41(-9.54, -5.28), <0.001	-4.50(-8.03, -0.96),0.013	-3.28(-4.37,-2.19),<0.001	-0.70(-3.42, 2.00), 0.61	-1.98(-4.35, 0.38), 0.10

Residence		Ref	Ref	Ref	Ref
Junju & Banda					
Ziani		1.47(-2.60, 5.55), 0.47	-0.51(-4.74, 3.72), 0.81	-4.98(-12.73, 2.76), 0.20	0.27(-2.62, 3.17), 0.85
Takaungu-Mavuani		1.70(-0.51, 3.93), 0.13	-0.80(-3.40, 1.80), 0.54	-5.68(-12.51, 1.15), 0.10	0.14(-2.60, 2.89), 0.91
Chasimba		1.11(-1.66, 3.88), 0.43	4.89(-4.08, 13.87), 0.28	-5.37(-12.31, 1.55), 0.12	-0.18(-2.75, 2.38), 0.88
Kilifi Township		-0.95(-2.68, 0.78), 0.28	-2.71(-5.81, 0.39), 0.08	-7.00(-13.81, -0.20), 0.04	-0.54(-3.09, 2.01), 0.67
Jaribuni & Kauma		-0.64(-3.77, 2.48), 0.68	-0.33(-3.84, 3.17), 0.85	-6.98(-13.80 -0.16), 0.04	-0.35(-2.96, 2.24), 0.78
Tezo		-0.47(-2.52, 1.60), 0.66	-1.94(-4.83, 0.94), 0.18	-7.40(-14.20, -0.59), 0.03	0.38(-2.39, 3.15), 0.78
Ngerenya		5.71(-2.09, 13.51), 0.15	-2.02(-5.64, 1.59), 0.27	-6.65(-13.55, 0.24), 0.06	-2.28(-4.88, 0.32), 0.08
Soko		7.31(-8.30, 22.92), 0.35	-3.26(-6.03, -0.49), 0.02	2.26(-13.77, 18.31), 0.78	0.02(-4.51, 4.56), 0.99
Roka & Gede		2.23(-4.88, 9.36), 0.53	-4.35(-6.76, -1.93), <0.001	-2.75(-13.65, 8.14), 0.62	-2.28(-5.32, 0.74), 0.14
					0.08(-15.50, 15.68), 0.99

Indicated values are coefficients (95% confidence intervals), P values of relative antibody concentrations per unit change in continuous covariate or compared to the reference group, for categorical covariates.

*Presence of parasites confirmed by PCR or microscopy during visits. Microscopy and PCR data for samples collected at birth (age, 0 months) were not available for this analysis.

5.3.2 Overall age-specific antibody prevalence

Age-specific antibody prevalence was calculated using antibody measurements obtained at all the three-monthly time points from birth up to the incidence of disease for cases (N=158) and corresponding controls (N=426). Overall, the highest antibody prevalence was against schizont extract (56.7%). Antigen-specific antibody prevalence was highest for AMA1 alleles (45% against AMA1 (FVO) and 46% against AMA1 (3D7)) whereas anti-*Pf*Rh2 antibodies were the least prevalent (19%). Antibody prevalence against MSP-2 (Dd2), MSP-2 (CH150/9), MSP-3 (3D7), MSP-3 (K1) and MSP-1₁₉ was 39%, 36%, 30%, 20% and 24%; respectively (Figure 5.1). Antibodies measured in cord blood samples were detectable in a significant proportion of children (94% and 93% for AMA1 FVO and 3D7 respectively, 88% and 85% for MSP-2 Dd2 and CH150/9 respectively, 69% and 66% for MSP-3 3D7 and K1 respectively, 46% for MSP-1₁₉ and 34% for *Pf*Rh2) but this decreased with increasing age, reaching a low plateau at 5-7 months for all antigens except AMA1 allelic types and *Pf*Rh2. Antibodies to AMA1 allelic types continued to decline until 11-13 months of age whereas those against *Pf*Rh2 reached a low plateau by the age of 4 months. Thereafter, a modest increase in antibody prevalence with age was observed for all antigens tested (Figure 5.1).

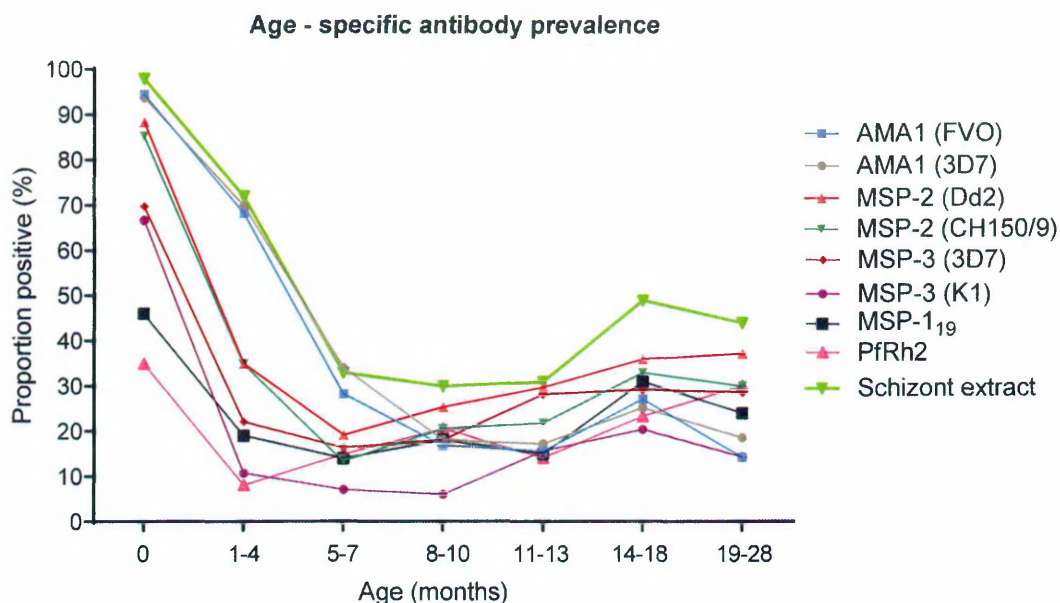


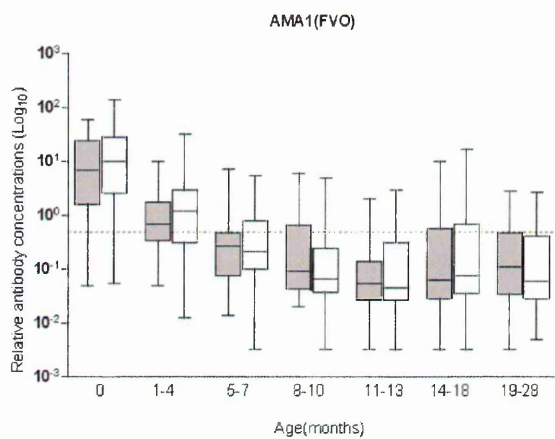
Figure 5.1 Age-specific antibody prevalence in children aged 0-2 years of age. Individual lines represent the antibody prevalence of the antigens tested across age categories grouped as; 0 months, 1-4 months, 5-7 months, 8-10 months, 11-13 months, 14-18 months and 19-28 months.

5.3.2.1 Age-specific antibody levels amongst the cases and controls

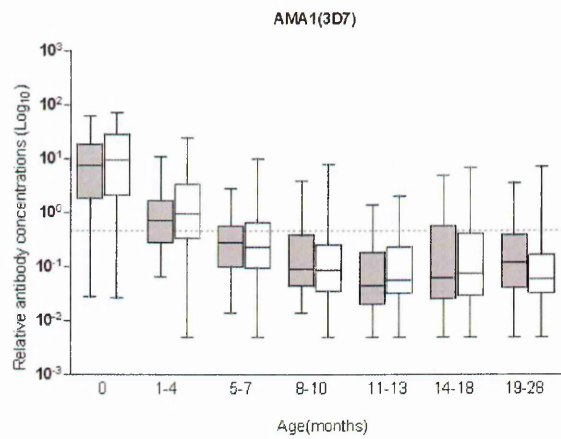
The highest antibody titres for all antigens tested were observed in cord blood plasma samples (Figure 5.2). There was a significant gradual decline in antibody titres with increasing age for all antigens tested (Kwallis test; $P=0.0001$) except MSP-1₁₉ (Kwallis test; $P=0.138$) and PfRh2 (Kwallis test; $P=0.225$). The decline in antibody titres reached a low plateau at 11-13 months for AMA1 allelic types, 8-10 months for MSP-2 allelic types, 5-7 months for MSP-3 (3D7) and 1-4 months for MSP-3 (K1), MSP-1₁₉ and PfRh2. For MSP-3 (K1), the low plateau was maintained upto 2 years of age, suggesting a lack of boost in antibody titres whereas for the other antigens there was a modest increase in antibody levels upto 2 years of age. Importantly,

there was no significant difference in median antibody titres between cases and controls across all the age categories analysed for all antigens tested (Figure 5.2).

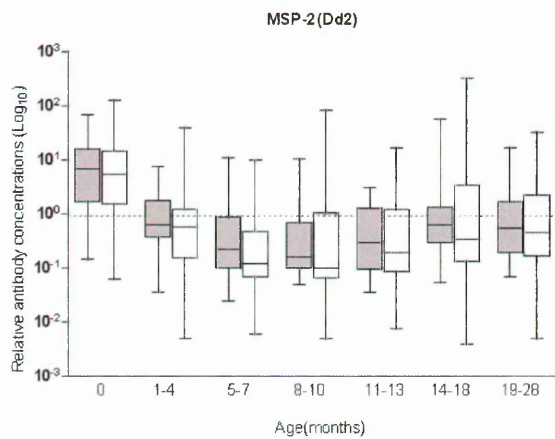
A.



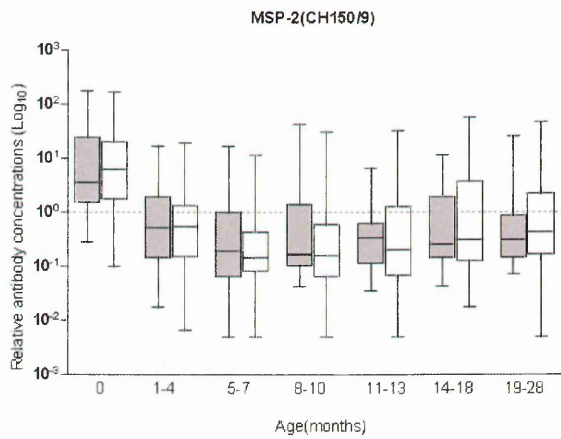
B.



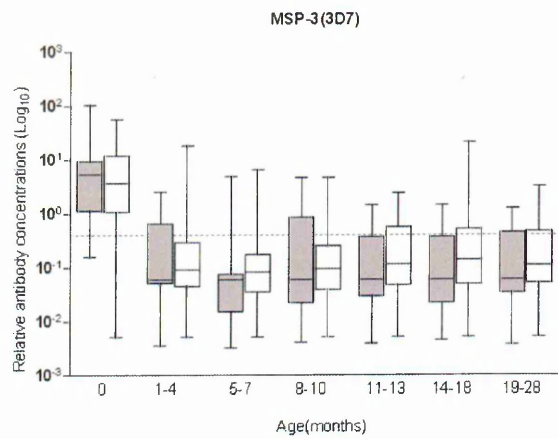
C.



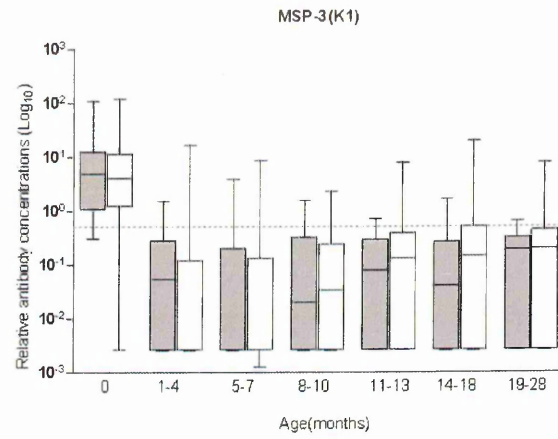
D.



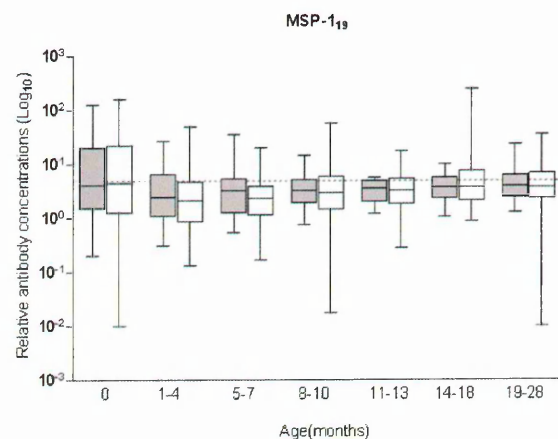
E.



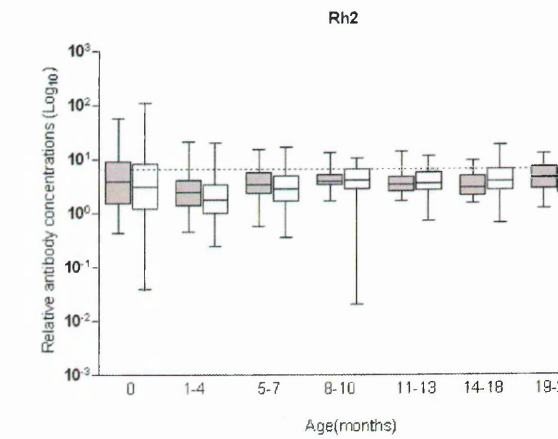
F.



G.



H.



I.

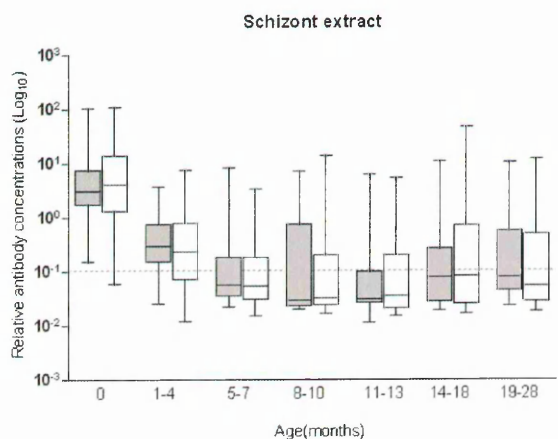


Figure 5.2 The level of antibodies to merozoite antigens by age in children aged 0-2 years. Box plots of relative antibody concentrations (in \log_{10} arbitrary units) against (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉, (H) *Pf*Rh2 and (I) schizont extract are shown for age categories 0 months, 1-4 months, 5-7 months, 8-10 months, 11-13 months, 14-18 months and 19-28 months. Antibody levels for cases (grey box plots) and controls (white box plots) at the different age categories are shown. Horizontal dotted lines represent the seropositivity cutoff defined as Mean+3SD antibody units of twenty European plasma samples.

5.3.3 Individual antibody profiles in relation to the presence or absence of infection

The profiles of antibodies were assessed in all children (both cases and controls) who had antibody and infection status data available at two or more sampling timepoints (N=190). There was considerable variability in the pattern of responses observed to some antigens, in particular against *Pf*Rh2 and MSP-1₁₉ (Figure 5.3). Consequently, the various profiles observed were grouped into five broad categories; (i) Children whose antibodies declined between 0-6 months (Figure 5.3 A-C); Children whose antibodies declined between 0-9 months but remained relatively stable thereafter (Figure 5.3 D-F); (iii) Children whose antibodies declined between 0-9 months but showed evidence of a boost in antibody titres to all antigens tested, in the presence of an infection (Figure 5.3 G-I); (iv) Children whose antibodies declined during the first 9 months of life but showed no evidence of a boost in antibody titres to all antigens tested, in the presence of an infection (Figure 5.3 J-L); and (v) children whose antibody titres declined between 0-9 months of age and showed evidence of boosting of antibody titres in the absence of a confirmed infection (Figure 5.3 M-O). In a large proportion of the children 72/190 (37%), there was evidence of declining antibody titres between 0-6 months of age, although in a few of the children the converse effect was observed, e.g. individuals 85, 116 and 132. Forty out of 195 (20%) of the children acquired an infection during the follow-up period, a proportion of whom either had evidence of boosting of antibody titres to all antigens tested (17/40, 42%), boosting of titres to particular antigens (12/40, 30%) or lack of boosting of antibody titres (11/40, 28%) to all antigens tested in the presence of an infection. Of particular interest was the observation that responses to at least one allele of MSP-2 were always boosted in the presence of an infection. There was also evidence of boosting of antibody titres in the absence of an infection in 47/190 (25%) of the children, although this observation could be explained by missing data on the infection status of 24/47 (51%) of individuals (Figure 5.3 M-O). In 36/190 (19%) of the children, antibody

titres declined during the first 6 months of life but remained stable over the follow-up period and there was no evidence of an infection in this group of children (Figure 5.3 D-F).

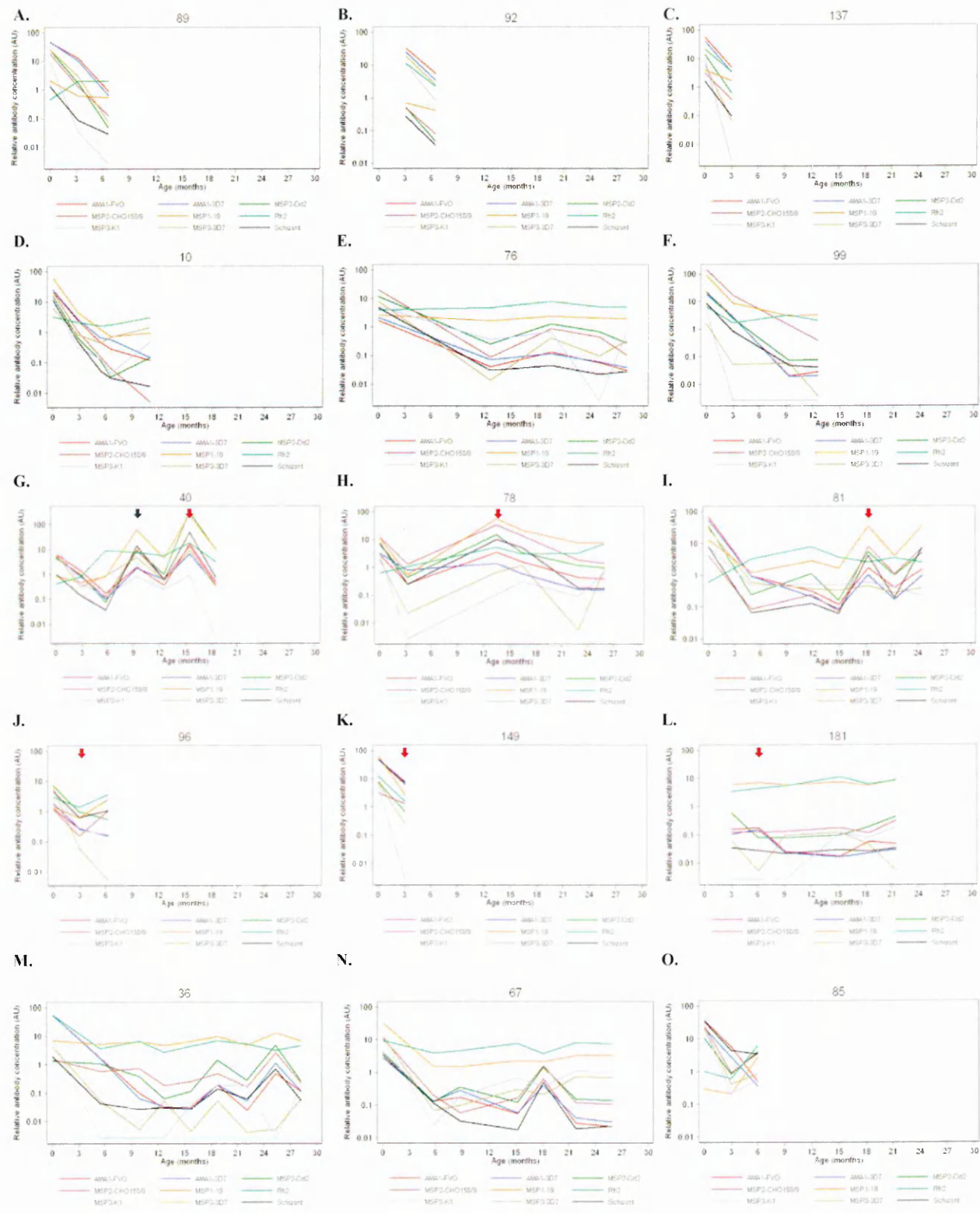
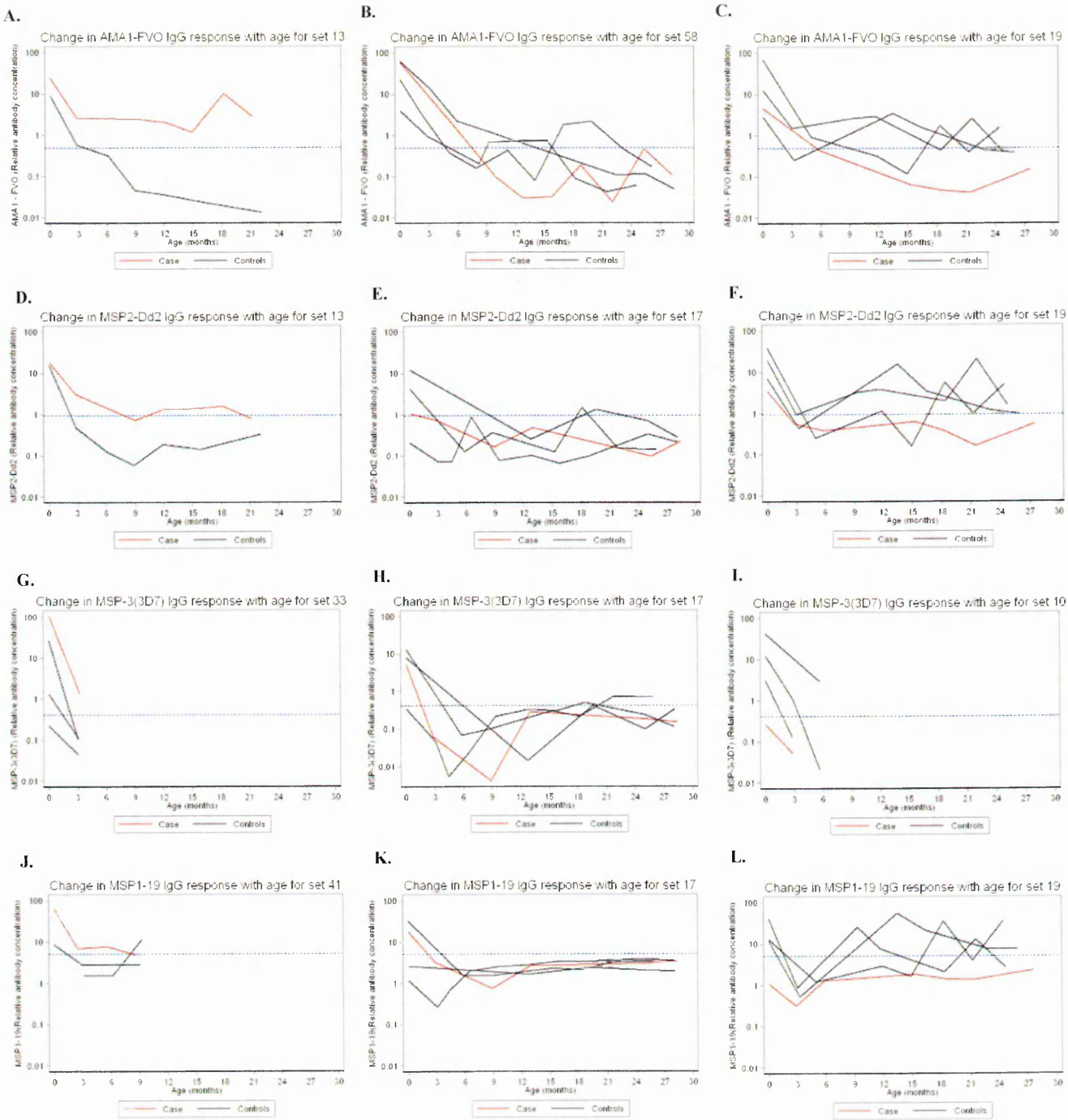


Figure 5.3 Individual profiles of IgG responses to merozoite antigens tested. Each panel represents the pattern of antibody responses observed in an individual during follow-up period. Individual lines represent responses to each antigen tested. The patterns of responses were grouped as follows: (A-C) Children with declining antibody titres between 0-6 months; (D-F) Children with declining antibody titres between 0-9 months and stable responses thereafter; (G-I) Children with declining antibody titres between 0-9 months followed by evidence of antibody boosting to all antigens tested in the presence of an infection; (J-L) Children with declining antibody titres between 0-9 months followed by no evidence of antibody boosting to all antigens tested in the presence of an infection; and (M-O) children with declining titres between 0-9 months followed by evidence of antibody boosting in the absence of an infection. Red, black and green arrows show presence of a *P. falciparum* infection confirmed by PCR only, PCR and microscopy, and microscopy only, respectively.

5.3.4 The profiles of antibody responses for individual risk sets

The longitudinal patterns of antibody responses were analyzed in individual risk sets. A risk set was defined as a group consisting of an index case and their individually matched controls. The profiles of antibody responses for individual risk sets were generated for 42 out of 61 (67%) risk sets following availability of data on antibody levels and infection status (Lundblom et al. 2013) at two or more consecutive sampling points. Three broad patterns of responses were observed for each risk set; (i) index cases maintaining relatively higher antibody titres over time compared to their matched controls (Group 1); (ii) index cases and controls maintaining similar antibody titres over time (Group 2); and (iii) index cases maintaining relatively lower antibody titres compared to their matched controls at the different sampling points (Group 3). Following previous reports showing that antibody responses are highly variable over time and are relatively short-lived in young children (Fonjungo et al. 1999, Kinyanjui et al. 2007, Akpogheneta et al. 2008), maintaining a relatively high antibody titre over the follow-up period was arbitrarily defined as achieving a titre above the cutoff point for seropositivity at more than 50% of the sampling points. As previously demonstrated in Figure 5.2, a majority of the risk sets had low antibody titres (below the seropositivity cutoff) at the different sampling points. There was a bias towards controls maintaining relatively higher antibody titres over time compared to cases (Group III responses) for AMA1 (FVO), MSP-3(3D7) and MSP-1₁₉ (52.4%, 47.6% and 42.9%; respectively) although these findings may be explained by the bias in comparing the pattern of responses of a single case against more than one control. The lowest proportion of risk sets (<20%) were classified into Group I (cases having higher antibodies compared to controls) for all antigens tested (Table 5.2).



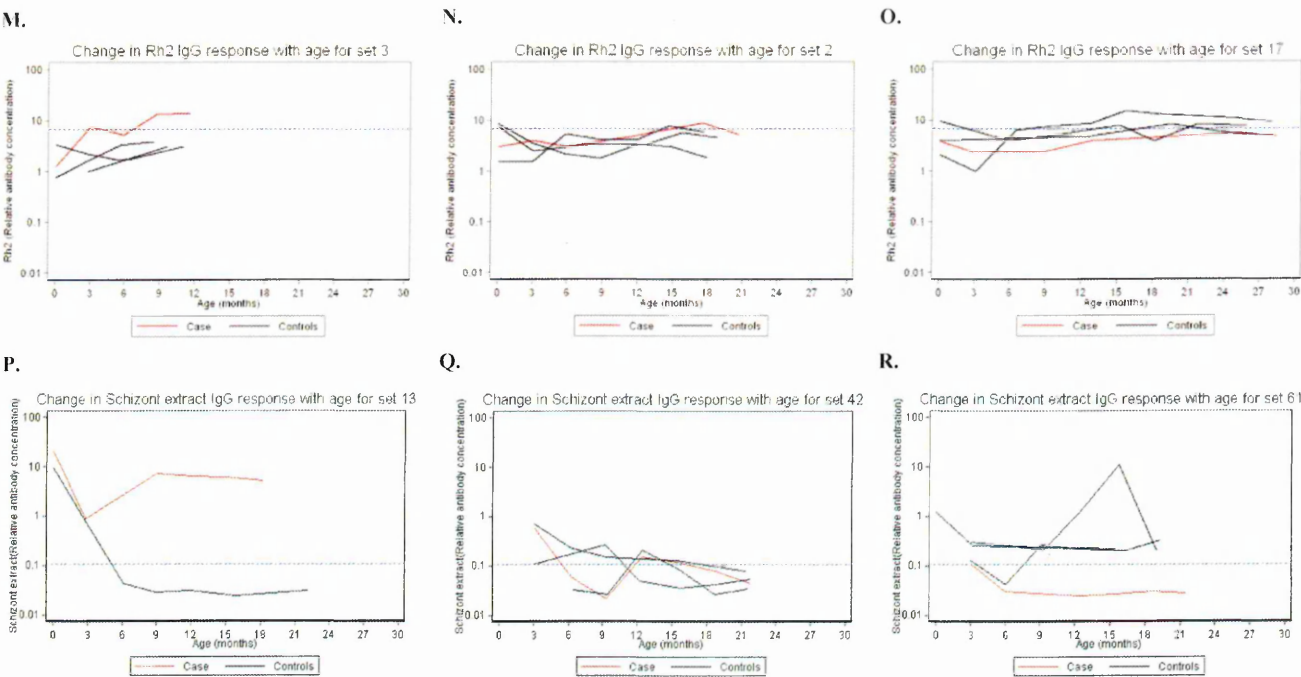


Figure 5.4. Representative patterns of IgG responses to merozoite antigens in defined risk sets. Each panel shows the profile of antibody responses in an individual risk set (defined as a group of an index case and individually matched controls). The three broad classifications of responses observed for individual antigens are shown in each column. Group I (panels A, D, G, J, M and P) represents antibody profiles where cases had relatively higher antibody responses over time compared to controls; Group II (panels B, E, H, K, N and Q) represents antibody profiles where antibody responses were similar in the cases and controls; and Group III (panels C, F, I, L, O and R) represents antibody profile where controls had relatively higher antibody responses over time compared to cases. Each row represents a specific antigen.

Table 5.2. The proportion of risk sets classified into three groups according to the profile of antibody responses for each antigen tested.

Antigen	Group I	Group II	Group III
AMA1 (FVO)	6 (14.3%)	14 (33.3%)	22 (52.4%)
MSP-2 (Dd2)	6 (14.3%)	22 (52.4%)	14 (33.3%)
MSP-3 (3D7)	7 (16.6%)	15 (35.7%)	20 (47.6%)
MSP-1 ₁₉	8 (19.0%)	16 (38.1%)	18 (42.9%)
PfRh2	5 (11.9%)	24 (57.1%)	13 (31.0%)
Schizont extract	6 (14.3%)	22 (52.4%)	14 (33.3%)

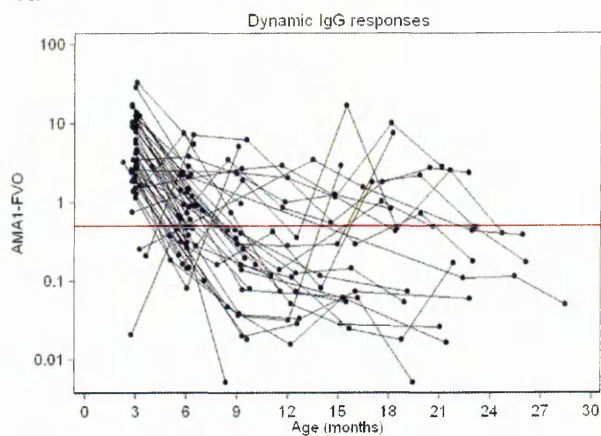
Total number of risk sets included in the analysis (N=42).

5.3.5 Dynamics of antibody responses over the follow-up period

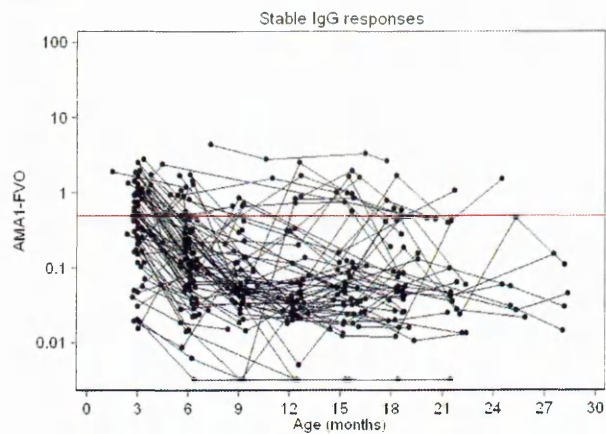
Although a large proportion of children had low antibody titres, often below the seropositivity cutoff at all time points, some children had detectable antibody levels that often fluctuated either above the cutoff (they were always seropositive but the actual levels varied) or around the cutoff (they were occasionally seropositive or seronegative at the different time points). Given this finding, I explored the maintenance of antibody responses over time to determine whether children generated either high or low levels of antibodies that were dynamic (highly fluctuant) or stable over time. Dynamic and stable responses were defined as achieving a standard deviation greater or less than 1; respectively, i.e. how far an individual’s antibody response fluctuated from their mean antibody response (Fowkes et al. 2012). This analysis was restricted to samples collected at >0 months to minimize the high standard deviation value

obtained when maternal antibodies were included in the analysis. With the exception of MSP-1₁₉ and *Pf*Rh2, a large proportion of children (>60%) had relatively stable antibody responses, often maintained below the seropositivity cutoff for all antigens tested (Figure 5.5.1 B, D, F, H, J and L). In contrast, a significant proportion of children classified as having dynamic responses had antibody titres fluctuating above the seropositivity cutoff, although this was less clear for MSP-1₁₉ and *Pf*Rh2 (Figure 5.5.1 A, C, E, G, I and K). Comparisons of dynamic antibody responses between the cases and controls revealed no significant differences between the proportions of responders in the two groups for all antigens tested (Figure 5.5.2) although there was a trend towards a higher proportion of dynamic responders amongst the control group for AMA1, MSP-3 alleles, *Pf*Rh2 and schizont extract compared to cases.

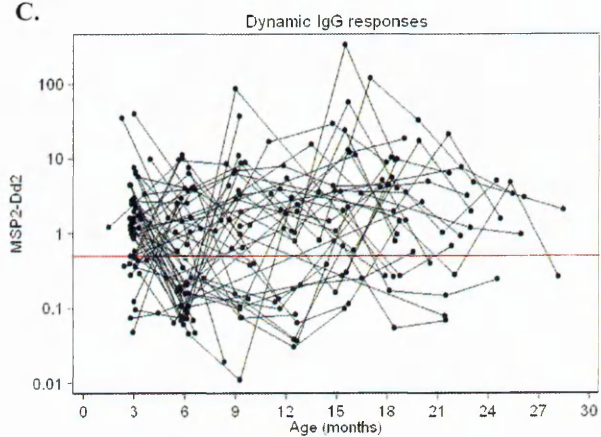
A.



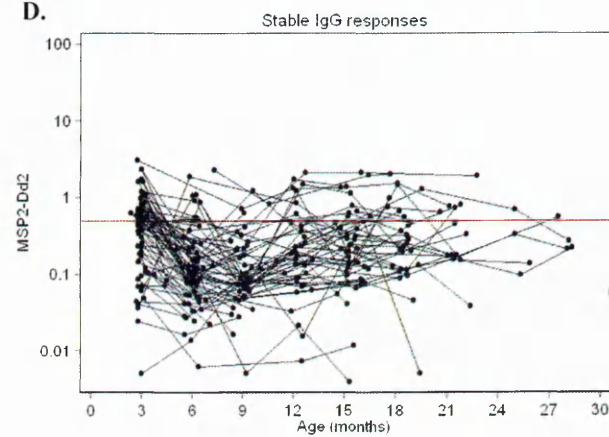
B.



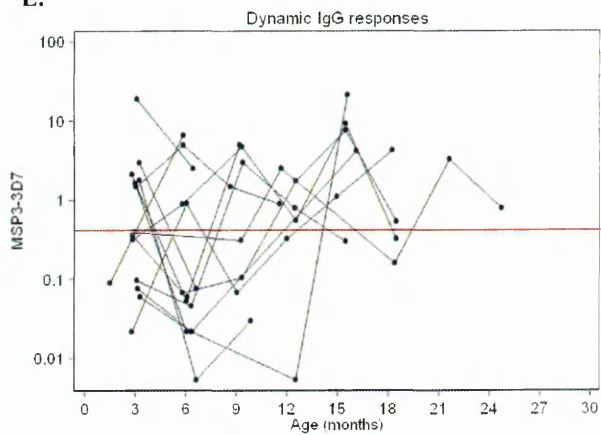
C.



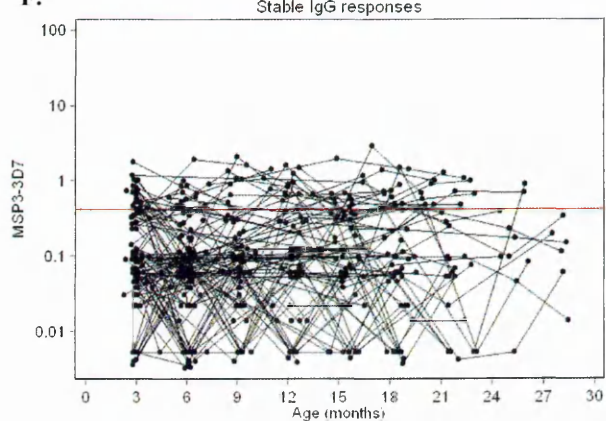
D.



E.



F.



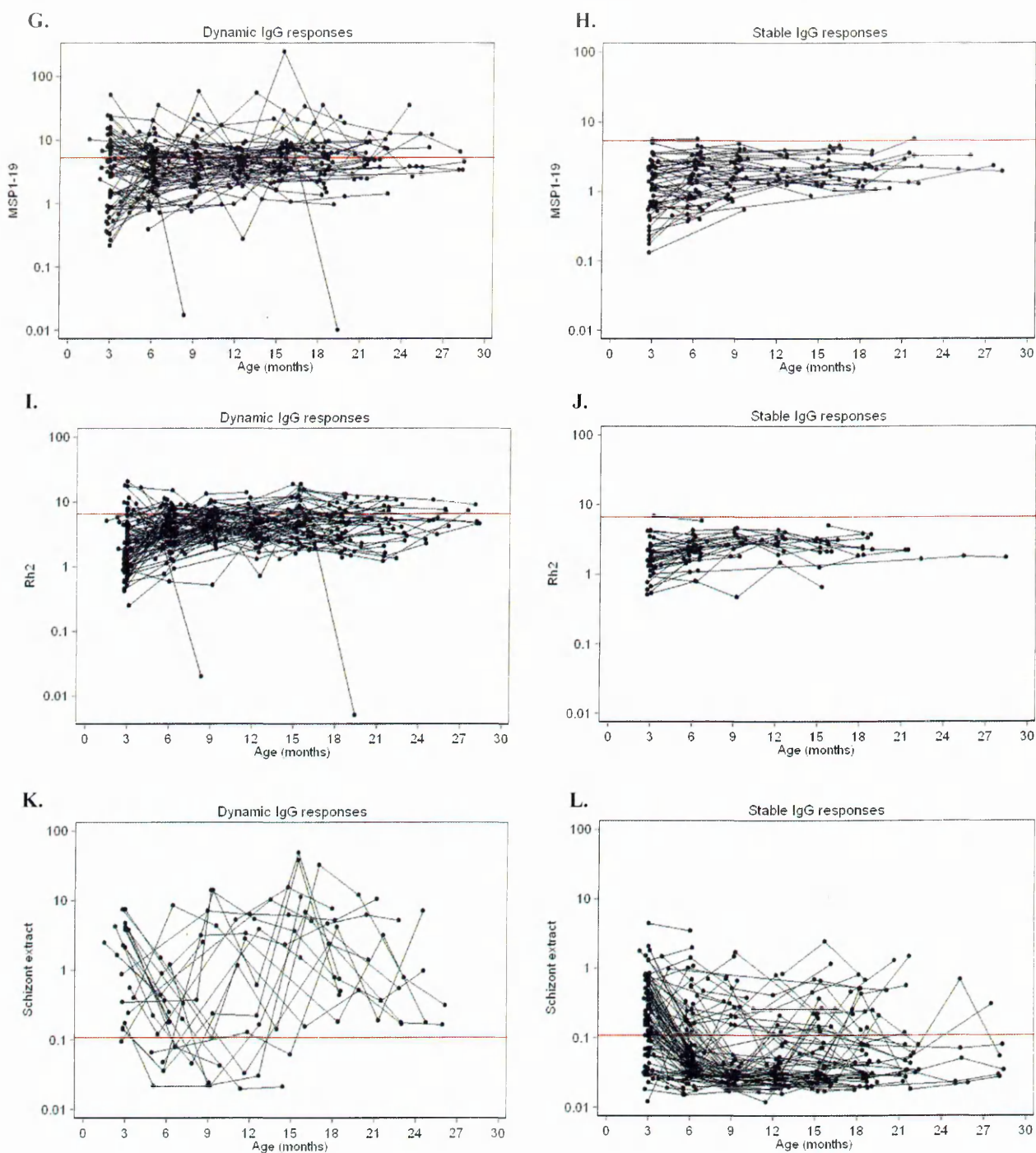


Figure 5.5.1. Antibody profiles over the follow-up period categorized as being relatively stable or dynamic. Representative graphs one allele of AMA1 (A-B), MSP-2(C-D), MSP-3(E-F), MSP-1₁₉ (G-H), *P*/Rh2 (I-J) and schizont extract (K-L) are shown. Horizontal red lines represent the seropositivity cutoff defined as mean+3SD of twenty European plasma. Stable and dynamic responses were defined as having a standard deviation less or greater than 1AU; respectively, i.e. how far an individual's antibody response fluctuated from their mean response.

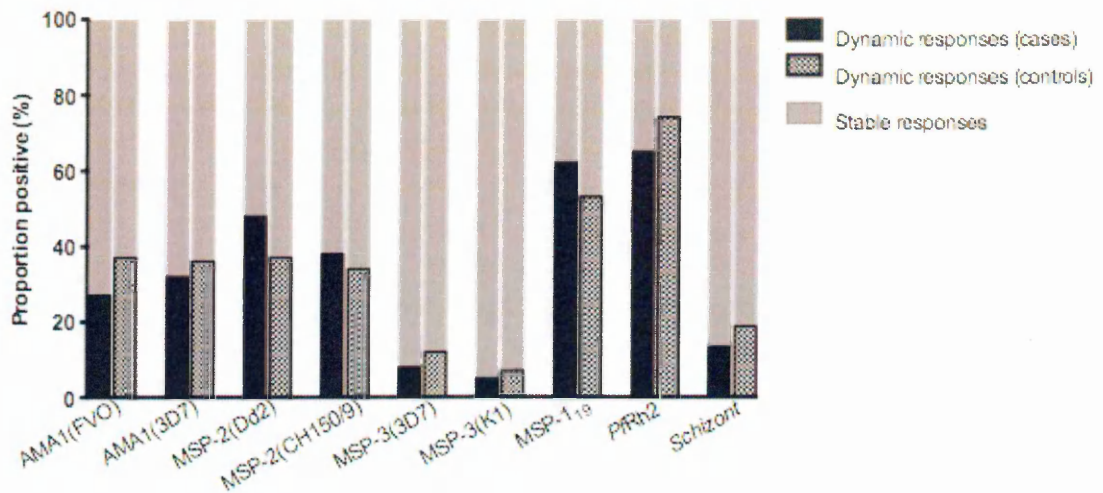


Figure 5.5.2. Proportion of cases and controls demonstrating dynamic versus stable responses over the follow-up period, to each antigen tested. Black bars and shaded bars represent the proportion of cases and controls with dynamic responses; respectively. Grey bars show the proportion of individuals with stable responses.

5.3.6 Association between antibodies and odds of developing severe malaria

The association between specific antibodies and the odds of developing severe malaria was assessed in the serum sample collected immediately prior to the disease episode (timepoint 1) or at consecutive three-monthly sampling time points (excluding antibodies measured at birth) for cases and corresponding controls as illustrated in Figure 5.6.

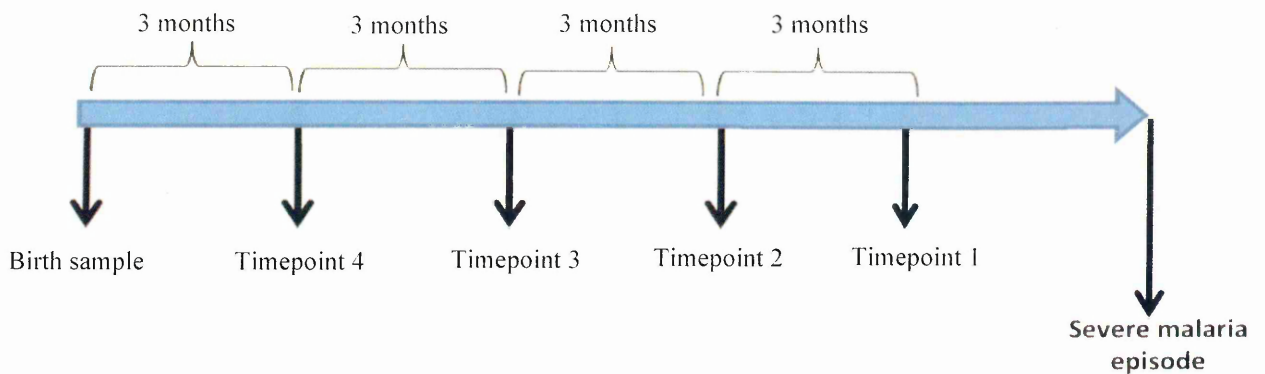


Figure 5.6 Illustration of the different time points that an antibody titre measurement was taken and used to calculate the protective efficacy of specific antibodies against severe malaria. Timepoint 1 and timepoint 4 represent the time points closest and farthest from the severe malaria episode, respectively.

The protective efficacy of specific pre-existing antibodies measured at the time point closest to admission for severe malaria cases and their corresponding controls are shown in table 5.3.1. As demonstrated in Figure 5.5.1, children classified as responders often had highly fluctuating antibody levels with levels dropping significantly by their subsequent follow-up visit. Previous studies have also demonstrated short-lived responses to blood-stage antigens in young children (Fonjungo et al. 1999, Kinyanjui et al. 2007, Akpogheneta et al. 2008) resulting in antibodies being more likely protective only within a limited duration of follow up. Taking this into consideration, separate analyses were conducted for i) all severe malaria admissions (N=53, median admission age 16.8 (range 5-74.06 months) and their corresponding controls (N=147, median age at case admission 18.2 (range 3.4-74.03 months)

and (ii) children admitted with severe malaria during the first 28 months of life (N=42, median admission age 10.76 (range 5-27.7 months)) and their corresponding controls (N=112, median age at case admission 9.5 (range 3.4-28.2 months)). In both analyses, only antibodies to AMA1 alleles were associated with a significant reduction in odds of developing severe malaria, (OR 0.39 (0.16-0.92) $p=0.03$ for AMA1 (FVO), OR 0.43 (0.19-0.99) $p=0.04$ for AMA1 (3D7) and OR 0.38 (0.16-0.90) $p=0.028$ for AMA1 (HB3)), in all severe malaria admissions. Similarly, antibodies to AMA1 (FVO and HB3) alleles were also associated with a significant reduction in odds of developing severe malaria in children followed up for 28 months from birth (OR 0.40 (0.17-0.98) $p=0.04$ for AMA1 (FVO) and OR 0.40 (0.16-0.98) $p=0.04$ for AMA1 (HB3)). Antibodies to all other antigens tested were not associated with protection from severe malaria (Table 5.3.1).

To determine whether antibody measurements obtained at multiple time points prior to the disease episode are better predictors of protection compared to those measured at the time point closest to the disease episode, mean antibody titres were calculated for each individuals' follow-up data, up to the incidence of disease for cases and their corresponding controls. Children whose mean antibody responses were above the seropositivity cutoff for each antigen were classified as responders. For most antigens tested, the antibody prevalence was overestimated based on the mean antibody response compared to the single time point measurement (53% versus 33% for AMA1 (FVO), 37% versus 27% for MSP-2(Dd2), 25% versus 21% for MSP-2(K1), 19% versus 5% for MSP-1₁₉ and 16% versus 11% for *Pf*Rh2, respectively). Antibodies to AMA1 alleles were associated with reduced odds of developing severe malaria in analyses including all children (OR 0.58 95% CI 0.30-1.13, $p=0.11$ and OR 0.77 95% CI 0.40-1.47, $p=0.43$, for FVO and 3D7 alleles, respectively) and in analyses including children admitted within 28 months of life (OR 0.61 95% CI 0.29-1.28, $p=0.20$ and OR 0.68 95% CI 0.33-1.41, $p=0.30$, for FVO and 3D7 alleles, respectively). All other antigens

tested were not associated with protection (Table 5.3.2). Strikingly, the odds ratio estimates obtained in analysis using the single time point closest to admission (Table 5.3.1) and using the mean response of multiple time points (Table 5.3.2) were similar in directionality.

Table 5.3.1. The protective efficacy of antibodies measured at one time point prior to admission, against severe malaria.

Antigen	All admissions (N=53)				Children admitted within 28months of life (N=42)			
	Univariate analysis	P value	Multivariate analysis*	P value	Univariate analysis	P value	Multivariate analysis*	P value
Schizont extract	1.45 (0.72-2.89)	0.29			1.13(0.52-2.47)	0.74		
AMA1(FVO)	0.54 (0.25-1.17)	0.12	0.39 (0.16-0.92)	0.03	0.53(0.23-1.18)	0.12	0.40(0.17-0.98)	0.04
AMA1(3D7)	0.55 (0.25-1.18)	0.12	0.43 (0.19-0.99)	0.04	0.55(0.24-1.24)	0.15	0.43(0.17-1.04)	0.06
AMA1(HB3)	0.52(0.24-1.12)	0.09	0.38(0.16-0.90)	0.03	0.51(0.22-1.17)	0.11	0.40(0.16-0.98)	0.04
MSP-2(Dd2)	1.13 (0.54-2.37)	0.72	0.99 (0.46-2.16)	0.99	1.22(0.54-2.78)	0.62	1.12(0.48-2.62)	0.78
MSP-2(CH150/9)	1.04 (0.47-2.30)	0.92	0.86 (0.36-2.02)	0.73	1.05(0.42-2.60)	0.90	0.92(0.36-2.36)	0.87
MSP-3(K1)	0.93(0.34-2.53)	0.90	0.82(0.29-2.30)	0.72	1.33(0.47-3.76)	0.58	1.16(0.39-3.40)	0.77
MSP-3(3D7)	1.36(0.59- 3.14)	0.46	1.26(0.53-2.97)	0.58	1.61(0.64-4.01)	0.30	1.44(0.56-3.70)	0.44
MSP-1 ₁₉	0.79 (0.14-4.25)	0.78	0.73 (0.13-3.92)	0.71	1.56(0.20-11.69)	0.66	1.50(0.20-11.00)	0.68
PfRh2	1.12 (0.47-2.67)	0.79	1.18 (0.49-2.88)	0.70	1.08(0.40-2.90)	0.86	1.12(0.41-3.05)	0.81

Estimates indicate the OR (95% CI).

*In multivariate analysis, reactivity to schizont extract (fitted as a continuous covariate) was taken into account, in the conditional logistic regression model due to the observed increased odds of exposure (as measured by schizont extract reactivity) in the cases compared to controls at this time point. These results suggested that there was residual confounding, despite the matching of cases to individual controls that was done to minimize this.

Table 5.3.2. Protective efficacy of the mean antibody titres of measurements obtained at multiple time point prior to admission, against severe malaria.

Antigen	All admissions (N=53)				Children admitted within 28months of life (N=42)			
	Univariate analysis	P value	Multivariate analysis*	P value	Univariate analysis	P value	Multivariate analysis*	P value
Schizont extract	1.07 (0.58-1.98)	0.23			1.17 (0.55-2.49)	0.68		
AMA1(FVO)	0.60 (0.31-1.16)	0.13	0.58(0.30-1.13)	0.11	0.63 (0.30-1.30)	0.21	0.61 (0.29-1.28)	0.20
AMA1(3D7)	0.79 (0.42-1.50)	0.48	0.77(0.40-1.47)	0.43	0.70 (0.34-1.43)	0.33	0.68 (0.33-1.41)	0.30
MSP-2(Dd2)	1.36 (0.70-2.61)	0.35	1.38 (0.71-2.70)	0.33	1.32 (0.63-2.74)	0.45	1.34 (0.63-2.84)	0.43
MSP-2(CH150/9)	1.47 (0.74-2.92)	0.26	1.46 (0.73-2.94)	0.28	1.34 (0.62-2.90)	0.44	1.31 (0.60-2.87)	0.49
MSP-3(K1)	1.20 (0.50-2.87)	0.67	0.94 (0.37-2.37)	0.90	1.42 (0.54-3.74)	0.46	1.10 (0.39-3.08)	0.84
MSP-3(3D7)	0.79 (0.29-1.66)	0.42	0.69 (0.29-1.66)	0.42	1.08 (0.45-2.58)	0.85	0.98 (0.39-2.43)	0.97
MSP-1 ₁₉	0.83 (0.35-1.94)	0.67	0.81 (0.34-1.92)	0.64	0.77 (0.28-2.11)	0.62	0.74 (0.26-2.04)	0.56
PfRh2	1.08 (0.41-2.86)	0.86	0.88 (0.31-2.50)	0.82	1.20 (0.40-3.59)	0.73	0.93(0.28-3.05)	0.91

Estimates indicate the OR (95% CI).

*In multivariate analysis, reactivity to schizont extract (fitted as a continuous covariate) as a proxy for previous cumulative exposure to infection was taken into account, in the conditional logistic regression model.

5.3.7 Association between the breadth of antibody responses and odds of developing severe malaria.

Previously, we have shown that the breadth and depth of anti-merozoite responses was associated with a reduced risk of developing clinical episodes of malaria (Osier et al. 2008). To demonstrate the role of breadth of antibody responses in protection from severe malaria as an outcome, the proportion of cases and controls responding to a given number of antigens (N=5) was calculated (Figure 5.7A). The only differences observed between the two groups were in the proportion of children responding to at least one antigen (16% versus 27%) and those responding to none of the antigens tested (44% versus 32%), in cases and controls respectively. Increasing breadth of antibody responses was associated with a stepwise increase in risk of developing severe malaria (Figure 5.7B). Responses to at least one antigen were associated with a significant decrease in risk of developing severe malaria (OR 0.36, 95%CI 0.13-0.98, $p=0.04$).

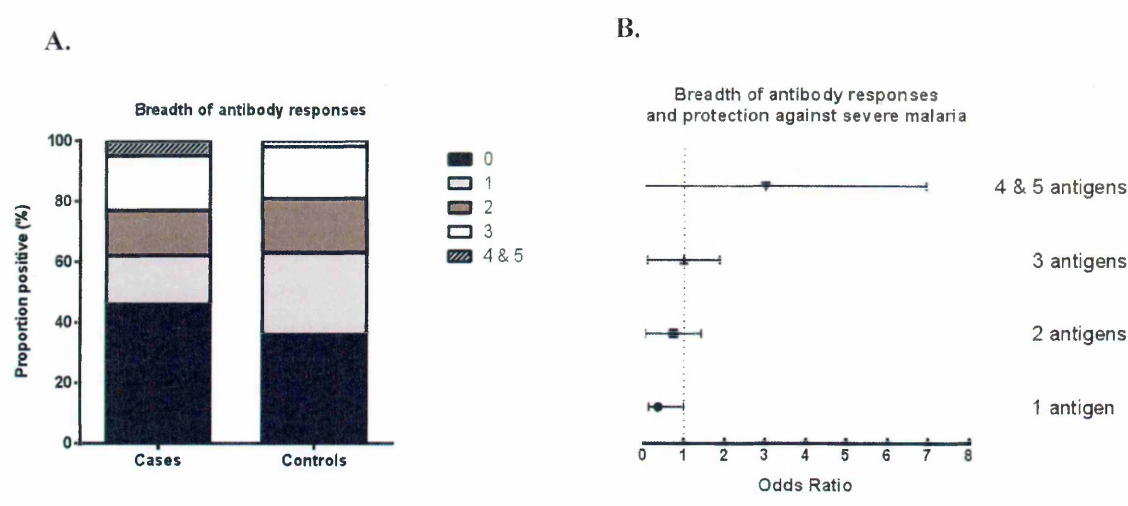


Figure 5.7 The breadth of anti-merozoite antibody responses in cases and controls (A). A total of five antigens (AMA1, MSP-2, MSP-3, *Pf*Rh2 and MSP-1₁₉) were tested. Black, light grey, dark grey, white and shaded bars represent the proportion of individuals responding to 0, 1, 2, 3 and >4 antigens respectively. (B) Protective efficacy of increasing breadth of antibody responses. The odds ratios and 95% CI are shown.

5.4 Discussion

Two major strengths of this study were, first, the availability of samples collected at multiple three-monthly intervals from birth upto 2 years of age to assess the cumulative contribution of an individual’s history and/or profile of antibody responses to protection from severe malaria. Contemporarily, pre-existing antibodies have often been measured at one time point prior to the disease episode and prospectively associated with the risk of developing malaria over a defined duration of follow-up, however this approach assumes that an individual’s ability to mount an antibody response can be potentially assessed at only one time point whereas for anti-malarial antibodies, the levels vary with exposure (Kinyanjui et al. 2009), they are

relatively short-lived in children (Kinyanjui et al. 2007, Akpogheneta et al. 2008) and their longevity varies depending on the antigen (Akpogheneta et al. 2008, Drakeley et al. 2005); hence some individuals may be incorrectly classified as being non-responders when sampled at a single time point. Thus far, to the best of my knowledge, no study has compared the protective efficacy of antibodies measured at a single time point versus the overall/mean antibody response for the same individual against episodes of severe malaria and this study therefore provides evidence for the first time that in this age-group of children, the odds of developing severe malaria conferred by antibodies measured at a single time point are predictive of the odds likely to be obtained based on an individual's overall pattern of responses. These findings suggest that sampling of antibodies at the time point immediately prior to the disease episode accurately reflects an individuals' overall antibody response. I interpret these findings with caution particularly because the majority of the children in this cohort had low and stable antibody responses over time such that a mean of cumulatively low antibody responses would be captured nonetheless at any single time point. In a separate study conducted in Kilifi in which two cross-sectional surveys of children aged less than ten years were carried out each year between 2002 and 2004, they showed that in the cross-sectional survey of May 2002, antibody responses to AMA1 (W2mef allele) were associated with an increased risk of developing uncomplicated malaria during the ensuing 6 months of follow-up and yet this was not the case in October 2002 and May 2003 possibly due to declining malaria transmission intensity during the period of their study. A longitudinal analysis of responses measured at all the sampling points revealed that anti-AMA1 (W2mef) antibodies were associated with a significantly increased risk of developing uncomplicated malaria during the follow-up period, a finding which is in contrast to what was observed in this chapter (Cleopatra Mugenyi, 2010, PhD Thesis). Although this study focused on responses to similar merozoite antigens and samples were also collected during a period of declining malaria

transmission, it is not possible to perform a head to head comparison between their findings and mine because of differences in the age ranges studied, the sampling framework, analytical methods and the clinical outcome of uncomplicated malaria that was evaluated.

Overall there was a lack of association between antibodies to the panel of merozoite antigens measured at multiple or at a single time point from birth with protection against severe malaria during the first two years of life, with the exception of AMA1 antibodies which were associated with a 40-50% reduced odds of developing severe malaria. Previous studies have looked at responses to schizont extract (Ndungu et al. 2002) and to infected erythrocyte surface antigens (Bull et al. 2002), in a broader age range of children (1-5 years) and showed that anti-schizont extract IgG1 antibodies were associated with a reduced risk of developing severe malaria whereas agglutinating antibodies against 3 isolates obtained from children admitted to hospital with malaria were not associated with protection from severe malaria, respectively. Although in the study by Ndungu *et al*, 2002, they demonstrated a role for IgG1 in protection against severe malaria as an endpoint of interest, it is difficult to demonstrate the targets of protective antibodies from the antigen specificities contained in a crude parasite lysate and my data therefore provides, for the first time, i) an assessment of a much broader panel of merozoite antigens in a longitudinally monitored birth cohort; and ii) included a much larger sample size of 61 severe malaria cases rather than the 21 assessed in the above studies. Findings from similar studies conducted in this region (Polley et al. 2004, Osier et al. 2008) and others (Stanisic et al. 2009, Gray et al. 2007) have shown that naturally-acquired antibodies against AMA1 are associated with protection against uncomplicated clinical episodes of malaria. Following the paucity of data on the targets of protective responses against severe malaria as a clinical endpoint, the findings presented in this chapter lend support for the development of an AMA1 vaccine which will not only protect against uncomplicated clinical episodes but also the severe form in the most vulnerable age group.

Antibody prevalence and titres were highest at birth but gradually decayed to a low plateau at 5-7 months of age for most antigens tested. Thereafter, antibody titres and prevalence were maintained at relatively low and stable levels with some evidence of a slow but steady increase in prevalence with age. This is consistent with findings from a birth cohort of Gambian (Duah et al. 2010) and Kenyan (Malhotra et al. 2009) children. The observation of low antibody prevalence in this group of children probably reflects inadequate exposure to infection due to young host age and the declining malaria transmission intensity in this area between 2002 and 2008 (Okiro et al. 2007, O'Meara et al. 2008). Only 19% of the children harbored asymptomatic infections during the six year follow-up period, an estimate which is significantly below the 40% cutoff proposed by some studies as the cross-sectional parasite prevalence rate in children aged 2-10 years ($PfPR_{2-10}$) that reflects a population under low malaria transmission intensity (Noor et al. 2009, Smith et al. 2007, Hay et al. 2008). In a malaria holo-endemic area in Western Kenya, antibody prevalence to MSP-1₄₂, AMA1 (FVO) and EBA175 was between 10% and 30% in children aged 6 – 36 months (Malhotra et al. 2009). In a separate study in the same area, Branch *et al.*, examined the pattern of responses of 24 infants from birth up to 1 year of age and showed that despite documented evidence of exposure to multiple infections, which was accompanied by peaks in MSP-1₁₉ antibody levels, the duration of antibody responses and overall antibody levels did not increase over time (Branch et al. 1998). I would argue that the findings from these studies and the data presented in this chapter suggest that, despite evidence of exposure to infection, there are intrinsic age-dependent factors that impair the ability of young children to mount high antibody titres to the panel of antigens tested in children of this age range. In contrast, a study conducted in a low altitude area in Tanzania argues against this notion by showing that MSP-1₁₉ antibody prevalence in children aged 12-24 months was approximately 70% (Drakeley et al. 2005), an

estimate that was significantly higher than what was observed in this thesis and in Western Kenya (Malhotra et al. 2009).

Despite the frequent low reactivity to individual antigens, antibody titres in this study were highly dependent on concomitant exposure to infection, consistent with previous studies (Proietti et al. 2013, Kinyanjui et al. 2004, Akpogheneta et al. 2008, al-Yaman et al. 1995). A positive association between asymptomatic infection, previous exposure (as measured by schizont extract reactivity), and year of sample collection with antibody titres was observed. Transient peaks in antibody titres that rapidly declined during the subsequent three-months of follow-up were noted among individuals that had a documented *P. falciparum* infection, similar to what has been observed in other studies (Branch et al. 1998, Fonjongo et al. 1999, Taylor et al. 1996, Cavanagh et al. 1998). It is plausible that the transient peaks derive from short-lived plasma cells generated from immature B cells rather than pre-existing long-lived plasma cells (LLPCs) or memory B cells (mBCs), following the observation that many of these children were experiencing their first infections and mounted antibody responses that rapidly declined following parasite clearance together with supporting data from mathematical models demonstrating inefficient generation of long-lived plasma cells in Ghanaian children aged 0-2 years (White et al. 2014a). Although the development of immune memory to malaria in children of a similar age group has so far not been studied, there is evidence that mBCs are efficiently acquired even following single infections in mice (Ndungu et al. 2009) and maintained in the absence of exposure (Ndungu et al. 2012, Wipasa et al. 2010).

I also observed diverse longitudinal patterns of responses in which some children had peaks in antibody responses to some antigens in the absence or presence of asymptomatic infections. These findings could be explained by several factors such as insufficient sampling for asymptomatic infections, the nature of the antigens (some antigens may be more

immunodominant than others), clonal imprinting (original antigenic sin) as seen in influenza infection (Wrammert et al. 2011), bystander polyclonal activation by mitogens (Bernasconi, Traggiai and Lanzavecchia 2002), host genetic differences, hemoglobinopathies or environmental factors. However, direct evidence for some of these factors remains elusive. In a study by Taylor *et al.*, they genotyped HLA class-II alleles in a population and showed that genetic differences did not play any role in explaining non-responsiveness to particular antigens (Taylor et al. 1996). There are considerable disparities between studies regarding whether IgG levels to merozoite antigens are lower (Sarr et al. 2006, Le Hesran et al. 1999, Miura et al. 2013a), higher (Diatta et al. 2004, Cabrera et al. 2005) or similar (Tan et al. 2011) in children with sickle cell trait compared to normal Hb. Further, it remains to be determined why antibodies to MSP-2 in the work presented here were preferentially boosted following infection as opposed to antibodies to other polymorphic and more immunogenic antigens such as AMA1 or a conserved antigen such as MSP-1₁₉. My observation is in contrast with two recent studies, one conducted in a low-transmission setting (Clark et al. 2012) and the other a controlled human malaria infection (CHMI) study that mirrors low dose, single exposure infections (Elias et al. 2014) that found preferential boosting of MSP-1₁₉-specific antibodies relative to other merozoite antigens.

Serological studies have provided evidence that the repertoire of targets of the antibody response increase with age and exposure, with the number of recognized antigens and magnitude of responses being correlated with age and immunity to uncomplicated episodes of malaria (Osier et al. 2008, John et al. 2005, Gray et al. 2007). Contrary to our previous finding that antibody breadth was associated with protection from uncomplicated malaria (Osier et al. 2008), the breadth of merozoite-specific antibody responses in this cohort was associated with a stepwise increase in risk of developing severe malaria. However, important differences between this study and our previous one were that: i) the age range studied was wider (1-10

years) and the highest proportion of children who had high titre responses to the least number of antigens were aged less than two years; ii) the antigens selected for inclusion in the breadth analysis in our previous study were individually associated with a reduced risk of developing clinical malaria, a result which was not replicated in this current study except against AMA1 alleles; iii) the number of recognized antigens was based on a cut off of high titre antibodies for each antigen in our previous study which could not be applied here since a large proportion of the children had low antibody levels; iv) the study was a cross-sectional survey at a specific time point with longitudinal monitoring for incidence of uncomplicated malaria episodes conducted during a fixed period of one transmission season whereas in the study presented here, sample collection and monitoring of children admitted to hospital with severe malaria was spread out over a period of eight years; and v) the study was conducted during a period of relatively higher malaria transmission intensity compared to the study presented here. One interpretation of the observed correlation between breadth of responses with increased risk of disease is that in this age group, the breadth of responses simply acts as a marker of exposure to infection but the level of exposure is insufficient and does not lead to induction of the adequate amounts of antibodies associated with immunity to malaria.

It is also clear that a proportion of children were less at risk of developing severe malaria compared to others despite the absence of merozoite-specific antibodies to the antigens studied here. The panel of merozoite antigens selected for this study was small and it remains plausible that there may be other potential targets of protective antibodies against severe malaria in young children. Such targets include antigens on the infected RBC surface which are plausible targets given the proposition that only a restricted subset of variants are expressed during severe malaria (Bull et al. 2005, Kaestli et al. 2006, Kyriacou et al. 2006, Jensen et al. 2004) to which antibodies are acquired more rapidly (reviewed in (Chan et al. 2014)). Towards this end, some studies have measured pre-existing agglutinating antibodies to

various infecting parasite isolates and demonstrated an association with protection against clinical malaria (Marsh et al. 1989, Dodo et al. 2001, Giha et al. 2000, Chan et al. 2014) or severe malaria (Bull et al. 1998) albeit inconsistently (Bull et al. 2002). Others have examined antibody responses to various domains of the most studied PfEMP1 antigen (Mackintosh et al. 2008b, Dodo et al. 2001, Magistrado et al. 2007) and also reported inconsistent findings. The contribution of other antibody unrelated factors should also be explored in future studies. For instance, we have demonstrated in this same cohort of children that asymptomatic infections detected at least once from birth during the three-monthly visits were associated with increased odds of developing severe malaria (Lundblom et al. 2013). Others have shown that prenatal exposure to parasite antigens could lead to acquisition of a tolerant phenotype characterized by low levels of pro-inflammatory cytokines and high levels of IL-10, which increases the risk of infection during infancy (Malhotra et al. 2009). A recent birth cohort study has also shown that IL-1 β levels are associated with a 42% reduction in risk of developing severe malaria during infancy (Kabyemela et al. 2013). These studies point towards an important role of pro-inflammatory cytokines, which are released by innate and adaptive immune cells in reduction of parasite densities and risk of developing severe malaria. However, increasing evidence points towards the timing and fine balance between the pro and anti inflammatory responses in clearance of parasites and prevention of inflammation-mediated pathology (reviewed in (Riley et al. 2006).

In conclusion, I found no association between antibodies to most of the antigens tested here with protection against severe malaria and this could be explained by insufficient antibody titres which were influenced by the combination of young host age and low transmission intensity during the period the study. However the finding that AMA1 antibodies could play a role in protection against severe malaria in infancy encourages the hope that a vaccine

administered early in life could protect the population most at risk of dying from severe malaria.

CHAPTER SIX

The role of transplacentally acquired antibodies in protection against infection and severe malaria during the first six months of life

6.0 Introduction

Infants living in malaria endemic areas are resistant to malaria during the first few months of life. There is evidence of a low incidence of clinical episodes of malaria (McGuinness et al. 1998, Klein Klouwenberg et al. 2005, Larru et al. 2009), low density asymptomatic parasitemia (McGuinness et al. 1998, Wagner et al. 1998) and relatively few cases of severe malaria (Snow et al. 1998). Susceptibility to clinical episodes of malaria and high density parasitemia increases gradually after the age of 18 weeks (Wagner et al. 1998) which suggests that protection may be due to innate factors present in the infant that gradually wane with age. Among the factors that potentially contribute to malaria resistance in infancy are maternal antibodies which are transferred *in utero* via an Fc receptor mediated pathway (Simister 1998) and reach peak levels during the third trimester of pregnancy (Simister 2003). IgG1 is transported more efficiently due to its high affinity for neonatal Fc receptors expressed on syncytiotrophoblast cells in the placenta (Costa-Carvalho et al. 1996, Malek 2003, Malek et al. 1996). Although several studies have demonstrated that malaria-specific antibodies measured in cord blood are present in high concentrations and correlate with antibodies present in maternal circulation (reviewed in (Riley et al. 2001)), the association between maternally transferred antibodies and protection against malaria during the first few months of life is inconsistent across studies (reviewed in (Riley et al. 2001) and Table 6.1 below).

Table 6.1. The role of transplacentally acquired antibodies in protection against malaria in infants.

Study	N	Sampling	Outcome	Antigen tested	Finding	Comments
(Deloron et al 1997)	154	Monthly visits for 6 months	Infection (Microscopy)	Schizont, Pfl55/RESA antibodies in the birth sample	IgG2 against schizont was protective	
(Hogh et al. 1995)	100	3 monthly samples from birth upto 1yr of age	Clinical malaria	Schizont, MSP-1 ₁₉ responses in cord blood samples	MSP-1 ₁₉ antibodies protective	Maternal abs* decrease by the age of 2 months.
(Branch et al. 1998)	60	Monthly intervals from birth upto 1 yr of age	Time to infection	MSP-1 ₁₉ responses in the maternal blood sample at delivery	MSP-1 ₁₉ abs protective	Peaks in response correspond to time points with parasitemia
(Achidi et al. 1996)	117	Longitudinally for 1 yr	Clinical malaria	Schizont extract, CSP, Pfl55 antibodies in the birth sample	No protection	
(Riley et al. 2000)	143	Monthly visits for 20 weeks 2 weekly ACD** for clinical malaria	Infection (PCR & microscopy) Clinical malaria	Schizont, MSP-2, AMA1, CSP, Pfl55, MSP-1 ₁₉ antibodies in the birth sample. IgG subclasses also tested	No protection (Abs a marker of risk of infection than protective immunity)	Maternal abs decrease by 22 weeks of age.
(Kitua et al. 1999)	198	Longitudinally for 1 yr	Infection	NANP, SPf66, MSP-1 ₁₉ antibodies in the birth sample	No protection	

* Abs – Antibodies, **ACD – Active Case Detection

This discrepancy may be explained by the varying duration of follow-up from birth adopted by different studies, ranging between 20 weeks (Riley et al. 2000) and one year (Hogh et al. 1995, Achidi et al. 1996), differences in antigens tested and differences in the primary outcomes examined (Table 6.1). However in at least two studies, MSP-1₁₉ antibodies measured at birth were consistently associated with a reduced risk of developing a clinical episode of malaria (Hogh et al. 1995) or delaying time to first infection (Branch et al. 1998), although in both studies children were followed up for up to one year of age, a duration over which maternal antibodies are unlikely to persist.

Susceptibility to clinical episodes of malaria has also been shown to increase as the level of fetal hemoglobin (HbF) decreases in circulation. This lends support to the hypothesis that HbF may play a role in protection against malaria during the first few months of life. In *in vitro* experiments, Pasvol *et al.* demonstrated a significant inhibition of parasite growth in red blood cells containing HbF in comparison to cells containing adult hemoglobin (HbA) (Pasvol, Weatherall and Wilson 1977). These findings were corroborated by experiments done in transgenic mice expressing fetal hemoglobin, a study which also demonstrated that impaired parasite growth occurs as a result of HbF resistance to digestion by malarial hemoglobins (Shear et al. 1998). However, in contrast to these findings, a recent study has shown that parasites grow efficiently in HbF containing erythrocytes but display altered expression of the major antigen expressed on the surface of the infected red blood cell (PfEMP1) and they propose a mechanism by which HbF acts cooperatively with maternal IgG leading to impaired binding of these cells to human microvascular endothelial cells (Amaratunga et al. 2011). This mechanism remains to be clearly demonstrated using cord blood IgG and erythrocytes obtained from infants born to mothers living in malaria endemic areas, given that in their study, Amaratunga *et al.*, used a pool of immune IgG obtained from adults resident in Mali and not maternal or cord blood samples.

In addition, there is evidence that p-aminobenzoic acid (pABA), a folate precursor that is required for parasite growth but is present at low levels in breast milk, limits growth of parasites in breastfeeding infants (Kicska et al. 2003, Kretschmar 1966). Presence of lactoferrin in breast milk which binds iron has also been shown to inhibit the *in vitro* growth of parasites (Kassim et al. 2000). Taken together, these studies demonstrate a potential independent role for non-antibody mediated protection against malaria during the first few months of life.

In contrast, there's a general trend towards lack of protection conferred by transplacentally acquired antibodies to a majority of merozoite antigens tested (Riley et al. 2001), although only few studies have evaluated the quality of the antibody response (Wilson et al. 2013, Duah et al. 2010) and none so far has examined the protective efficacy of these antibodies taking into account recent evidence that the breadth (Osier et al. 2008) and concentration (Osier et al. 2008, Murungi et al. 2013) of merozoite-specific antibodies may be more important in protection against clinical episodes of malaria or infection than responses to single antigens or the mere presence of these antibodies in cord blood, assessed using seropositivity cutoffs. Besides, no study has systematically examined the kinetics of decay of maternal antibodies to merozoite antigens or evaluated their role in protection against the severe form of malaria during infancy.

6.1 Objectives

1. To determine the kinetics of decay of maternal antibodies against a panel of merozoite antigens.
2. To compare pristine antibody levels between children who subsequently acquired an asymptomatic infection and those who remained uninfected during the first six months of life.
3. To compare pristine antibody levels in children who subsequently developed severe malaria and those who did not, during the first six months of life.
4. To determine the protective efficacy of specific-antibodies against acquisition of asymptomatic infections and development of severe malaria during the first six months of life.

6.2 Methods

6.2.1 Serum samples

A total of one hundred and thirty cord blood samples were collected from mothers who delivered their infants at Kilifi District Hospital between 2001 and 2006. The infants were recruited into a birth cohort and followed up every 3 months until 2 years of age. During the three-monthly visits, a blood sample was collected, separated into plasma and RBC pellet fractions and stored at -80°C for ELISA and PCR assays, respectively.

6.2.2 Recombinant antigens and Multiplex ELISAs.

IgG levels to a panel of recombinant merozoite antigens; AMA1 (FVO and 3D7 allelic types), MSP-2(Dd2 and CH150/9 allelic types), MSP-3 (3D7 and K1 allelic types), *Pf*Rh2 and MSP-

1₁₉ were measured using a multiplex bead-based ELISA platform, as previously described in Chapter two. IgG responses to schizont extract were measured using a standard ELISA technique, as previously described (Ndungu et al. 2002).

6.2.3 Statistical analysis

All analyses were performed in Stata 11.0 (StataCorp, Texas, USA) and GraphPad prism 6 (GraphPad Software, Inc). A linear regression model was used to determine the influence of maternal age, parity, birth weight, year of birth, residential location and cumulative exposure to malaria infection (reactivity to schizont extract) on the levels of specific-antibodies. The Mann–Whitney U test was used to compare antibody titres measured at birth between children who prospectively acquired an infection and those who remained uninfected during six months of follow-up from birth. The rate of antibody decay was determined using a longitudinal mixed-effects model with a random intercept and slope component (Amanna, Carlson and Slifka 2007, Fowkes et al. 2012). The model accounts for repeated measurements per individual and was fitted to the log₁₀ transformed values of the antibody titres collected at age 0, 3 and 6 months. Maternal antibodies were expected to decay following an exponential decay model represented by the equation;-

$$L_t = L_0 e^{-kt}$$

Where L_t and L_0 are the antibody concentrations at age t and at birth; respectively, k represents the decay rate constant. Half-life estimates were calculated using the equation:

$$T_{1/2} = \ln 2 / k,$$

Where $T_{1/2}$ is the estimated half-life and k is the slope component of the mixed-effects model (Kinyanjui et al. 2007, Fowkes et al. 2012, Amanna et al. 2007, Ochola et al. 2009).

A logistic regression model was used to calculate the association between specific-antibodies and protection against acquiring asymptomatic infections or developing severe malaria during the first six months of life. Antibodies were fitted as categorical variables in the model based on two different cutoffs: (i) seropositivity cutoff defined as ELISA OD value above the mean + 3SD of twenty European plasma; and ii) threshold concentrations defined in chapter four (Murungi et al. 2013) and by Rono *et al.* (Rono et al. 2013).

6.3 Results

6.3.1 Descriptive analyses of mothers and infants at enrollment

The baseline characteristics of mothers and their newborns at enrollment are shown in Table 6.2. The mean maternal age and number of previous pregnancies were 26.67 years (IQR 14.77-48.03) and 4 (range 0-13), respectively. The mean birth weight was 2.79 kg (range 1.63 – 4.36) and the majority of the mothers were residents of Kilifi township area (34%) and Takaungu location (21.5%). The proportion of male to female births was similar (Table 6.2).

Table 6.2 Baseline characteristics of mothers and infants at enrollment

Maternal age (N=128), mean (range)	26.67 (14.77 – 48.03)
No. of previous pregnancies (N=92), mean (range)	4 (0 -13)
Sex female, n/N (%)	62/130 (47.7%)
Birth weight (N=66), mean(range)	2.79 (1.63 – 4.36)
Residence, n (%)	
Banda ra salama, Junju	10(7.6)
Ziani	5(3.8)
Takaungu	28(21.5)
Chasimba	15(11.5)
Kilifi township, Konjora	34(26.1)
Jaribuni, Kauma	4(3.1)
Tezo	20(15.3)
Ngerenya, Sokoke, Roka	14(10.7)

6.3.2 Factors that influence the level of antibodies measured in cord blood.

Among the factors examined that influence the levels of specific-antibodies measured in cord blood, only antibody titres to schizont extract was associated with a significant increase in antibody levels to the antigens tested ($p < 0.05$) (Table 6.3). A significant positive correlation between antibody titres to specific antigens and schizont extract was also observed, with the exception of responses to *PfRh2* which showed a weak positive correlation (correlation coefficients; 0.70, 0.74, 0.69, 0.51 and 0.22 for AMA1(3D7), MSP-2(Dd2), MSP-3(3D7), MSP-1₁₉ and *PfRh2*, respectively). Maternal age, birth weight, parity, year of birth and

residence of the mothers did not significantly influence the levels of specific antibodies measured in cord blood (Table 6.3)

Table 6.3. Factors that influence the levels of antibodies measured in cord blood samples against a panel of merozoite antigens.

Factors that influence cord blood antibody titers		AMA1(3D7)	MSP2(Dd2)	MSP3(3D7)	PfPRh2	MSP-1 ₁₉
Schizont extract antibody levels		0.63(0.46-0.81), <0.001	0.62(0.48-0.77), <0.001	0.65(0.49-0.81), <0.001	0.26(0.11-0.41), 0.001	0.80(0.61-0.99), <0.001
Mother's age		-0.01(-0.03, 0.01), 0.19	0.01(-0.01, 0.02), 0.29	0.01(-0.0001, 0.03), 0.05	0.0001(-0.01, 0.01), 0.98	0.007(-0.01, 0.02), 0.53
Birth weight		-0.10(-0.40, 0.20), 0.51	0.01(-0.26, 0.30), 0.90	-0.06(-0.37, 0.25), 0.70	0.10(-0.18, 0.38), 0.47	-0.04(-0.47, 0.38), 0.83
No. of pregnancies						
Primigravidae	Ref		Ref	Ref	Ref	Ref
Multigravidae		-0.24(-0.73, 0.25), 0.33	0.23(-0.21, 0.68), 0.29	-0.008(-0.51, 0.50), 0.97	-0.23(-0.65, 0.18), 0.26	0.10(-0.49, 0.70), 0.73
Year of Birth						
2002	Ref		Ref	Ref	Ref	Ref
2003		-0.10(-0.46, 0.24), 0.55	0.05(-0.25, 0.36), 0.72	0.01(-0.31, 0.35), 0.91	-0.03(-0.31, 0.23), 0.77	0.15(-0.25, 0.57), 0.45
2004		-0.11(-0.44, 0.22), 0.52	-0.15(-0.44, 0.13), 0.30	-0.04(-0.35, 0.26), 0.78	-0.02(-0.28, 0.22), 0.82	0.04(-0.34, 0.42), 0.83
2005		-0.14(-0.81, 0.53), 0.69	-0.28(-0.87, 0.29), 0.33	-0.26(-0.89, 0.36), 0.40	0.04(-0.47, 0.56), 0.85	-0.21(-1.00, 0.56), 0.58
2006		0.01(-1.12, 1.13), 0.98	-0.30(-1.28, 0.67), 0.53	-0.84(-1.89, 0.21), 0.11	0.35(-0.51, 1.21), 0.42	0.09(-1.21, 1.41), 0.88

Residence		Ref	Ref	Ref	Ref	Ref
Junju & Banda						
Ziani		0.32(-0.49, 1.15), 0.43	-0.27(-0.94, 0.39), 0.41	-0.21(-0.97, 0.54), 0.57	0.54(-0.11, 1.21), 0.10	-0.30(-1.26, 0.64), 0.52
Takaungu-Mavueni		0.45(-0.09, 1.01), 0.10	0.10(-0.34, 0.55), 0.65	-0.01(-0.51, 0.50), 0.98	0.19(-0.25, 0.63), 0.39	0.05(-0.58, 0.69), 0.85
Chasimba		0.21(-0.40, 0.82), 0.49	-0.09(-0.59, 0.40), 0.70	-0.26(-0.83, 0.29), 0.34	0.21(-0.27, 0.70), 0.39	-0.73(-1.44, -0.02), 0.04
Kilifi Township		-0.16(-0.71, 0.37), 0.53	-0.59(-1.03, -0.15), 0.009	-0.61(-1.11, -0.12), 0.01	0.10(-0.33, 0.53), 0.63	-0.49(-1.11, 0.13), 0.12
Jaribuni & Kauma		0.06(-0.83, 0.95), 0.89	0.06(-0.66, 0.78), 0.86	-0.41(-1.23, 0.40), 0.32	0.08(-0.62, 0.80), 0.80	-1.17(-2.19, -0.14), 0.03
Tezo		0.03(-0.54, 0.61), 0.90	-0.41(-0.88, 0.06), 0.08	-0.68(-1.22, -0.14), 0.01	0.27(-0.19, 0.73), 0.25	-0.25(-0.93, 0.41), 0.44
Ngerenya		-0.42(-1.13, 0.29), 0.24	-0.64(-1.22, -0.06), 0.03	-0.74(-1.40, -0.09), 0.02	0.05(-0.52, 0.62), 0.86	-0.76(-1.58, 0.06), 0.07
Soko		0.01(-1.15, 1.18), 0.98	-0.78(-1.73, 0.16), 0.10	0.17(-0.89, 1.25), 0.74	0.48(-0.44, 1.42), 0.30	-1.15(-2.49, 0.19), 0.09
Roka & Gede		-0.25(-1.14, 0.63), 0.57	-1.24(-1.97, -0.52), 0.001	-0.64(-1.46, 0.17), 0.12	0.03(-0.67, 0.75), 0.91	-0.69(-1.72, 0.33), 0.18

Indicated values are coefficients (95% confidence intervals), P values of the change in relative antibody concentrations per unit change in continuous covariate or compared to the reference group, for categorical covariates. Coefficient less than, greater than or equal to zero indicate a decrease, increase or no overall change in antibody concentrations per unit increase in explanatory variable, respectively.

6.3.3 Proportion of children acquiring asymptomatic infections or developing severe malaria during the study period.

Among the children that had a cord blood and three-monthly follow-up samples obtained (N=130), *Pf* infections and severe malaria cases were recorded in 27/130 (20.7%) and 25/122 (20.5%) children, respectively, during two years of follow-up from birth. Out of the 25 children who developed severe malaria, 9 of them had experienced previous asymptomatic infections. The number of children with a documented infection or developing an episode of severe malaria during the first six months of life was 10/130 (7.6%) and 5/130 (4.1%), respectively (Figure 6.1). Only one out of the five children who developed severe malaria during the first six months of life had evidence of a previous asymptomatic infection.

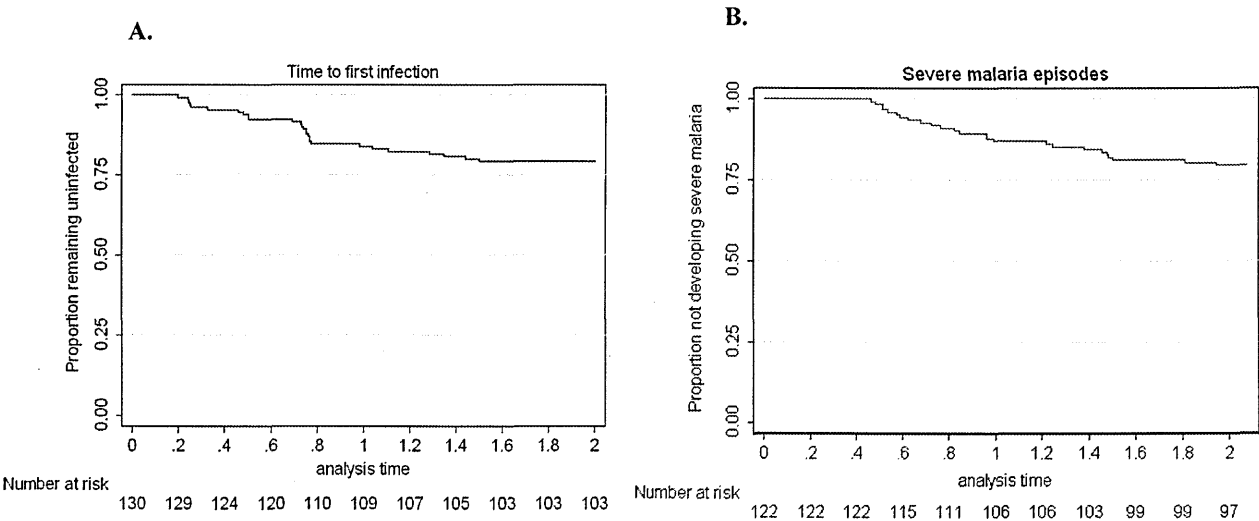
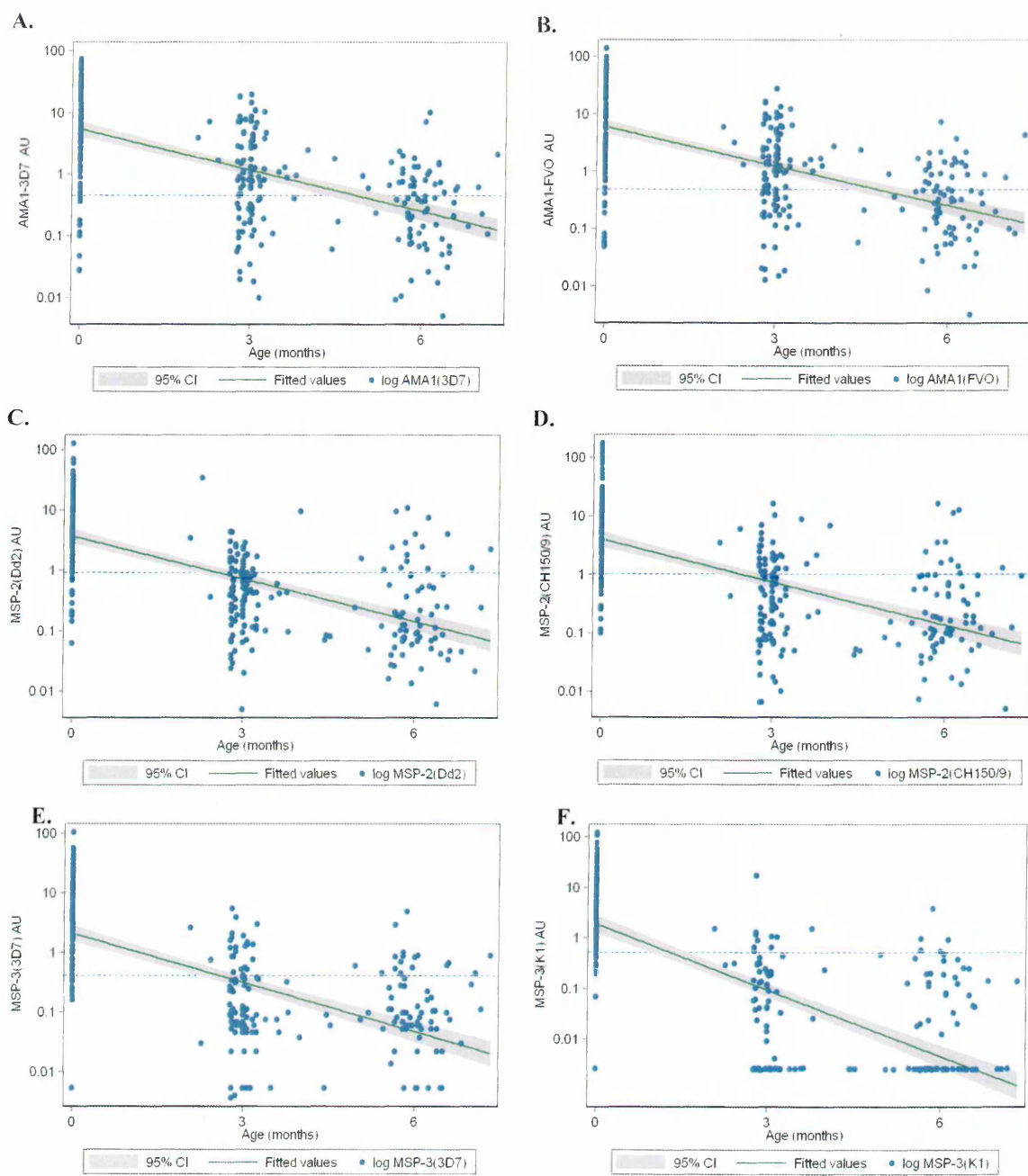


Figure 6.1 Kaplan-Meier curves of (A) time to first infection and (B) time to severe malaria episode in children who were recruited at birth and had a cord blood sample taken.

6.3.4 Decay of transplacentally acquired antibodies

The decay rate of antibodies to specific antigens was determined using a longitudinal mixed-effects model (Amanna et al. 2007, Fowkes et al. 2012). A regression line was fitted through \log_{10} transformed antibody titres for children aged less than six months. Antibody levels decreased exponentially with increasing age, as shown in Figure 6.2. Excluding children who experienced an infection, which is known to alter the natural decay of antibodies by boosting antibody levels, did not alter the overall decay rate in this population. This finding is expected given that only 10 out of 130 (7.6%) infants acquired infections in less than six months of age. Analysis of the overall rate of decay and duration of persistence of maternally transferred IgG revealed a relatively longer half-life for *Pf*Rh2 and MSP-1₁₉ antibodies (34.65 and 10.5 months, respectively) compared to other antigens tested (3.01, 3.12, 2.89, 2.80, 2.52, 1.53 months for AMA1(FVO), AMA1(3D7), MSP-2(Dd2), MSP-2(CH150/9), MSP-3(3D7) and MSP-3(K1), respectively) (Table 6.4). The antibody decay rates for individual infants followed a normal distribution with some infants exhibiting very rapid declines, some very slow and majority had decay rates around the mean estimate. There was minimal inter-individual variation in the rate of decline of specific-antibodies as illustrated by the narrow confidence intervals (Table 6.4); with the exception of antibodies to *Pf*Rh2 and MSP-1₁₉ which showed relatively wide variations in antibody half-life estimates (half-life was 10.5 months (7.45 – 17.32) for anti- *Pf*Rh2 antibodies and 34.6 months (16.90 – 866.25) for anti- MSP-1₁₉ antibodies).



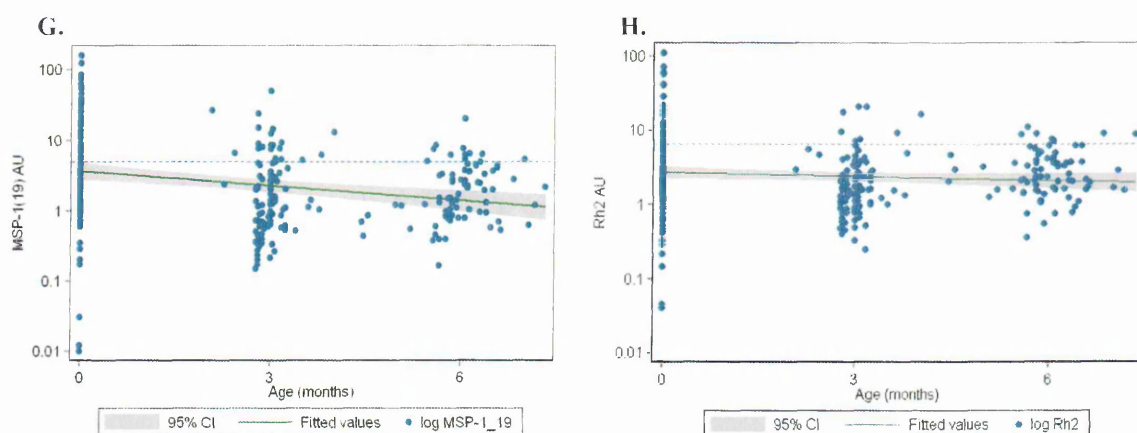


Figure 6.2 Scatter plots of antibody titres to (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) PfRh2 during the first six months of life. The fitted slope and 95% CI are shown. The dotted blue line represents the cutoff for antibody seropositivity defined as mean + 3SD of 20 European plasma.

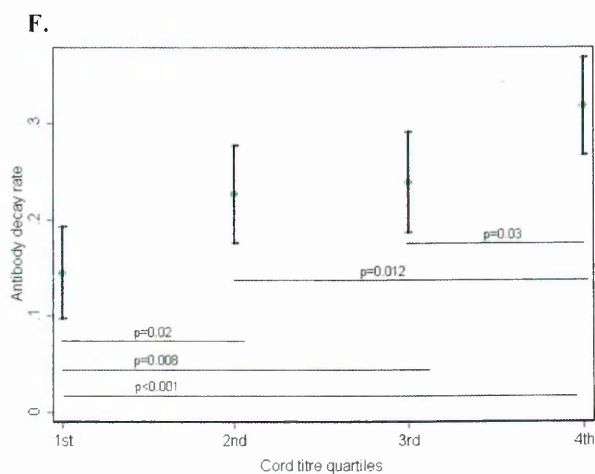
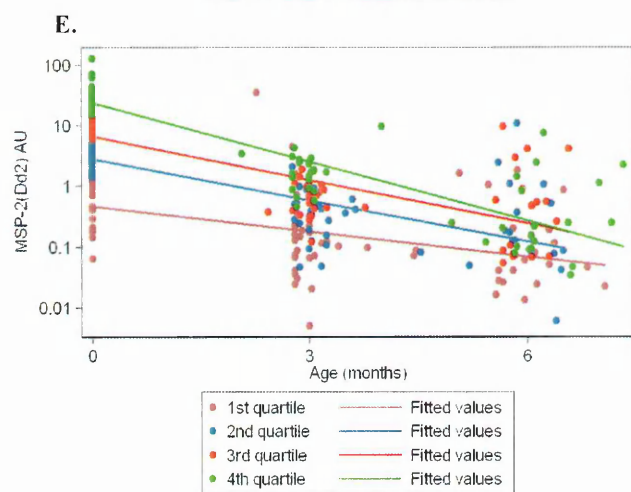
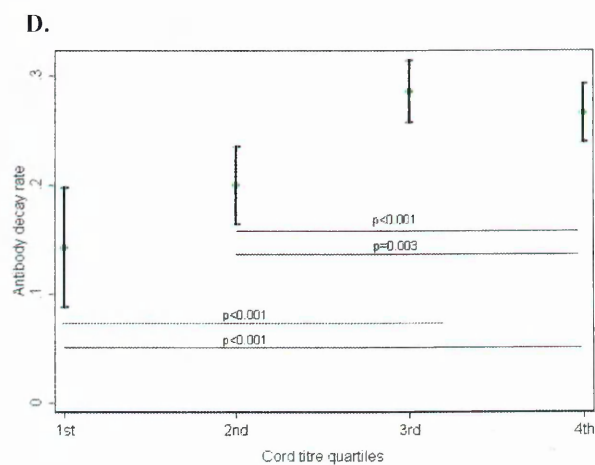
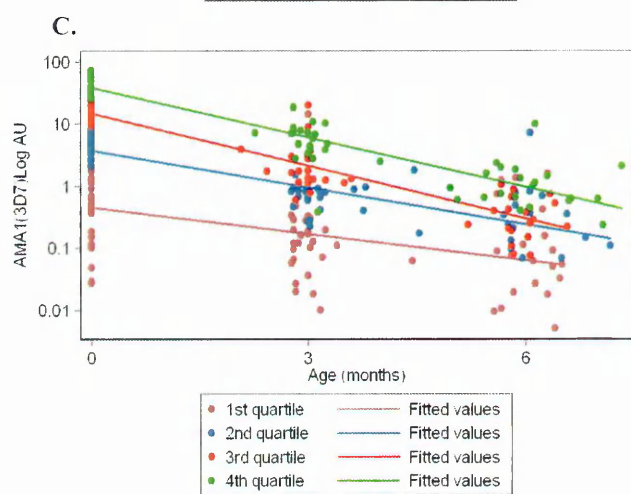
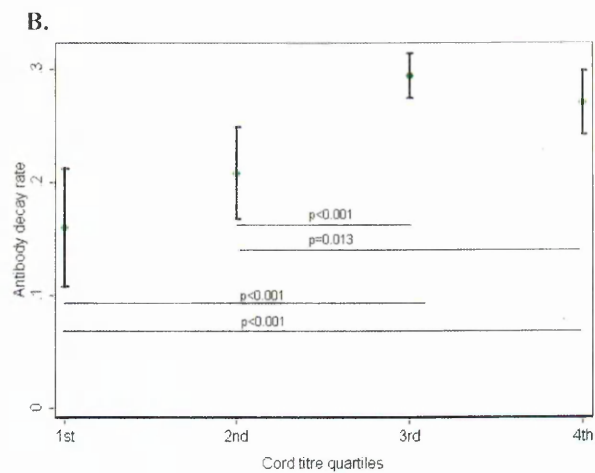
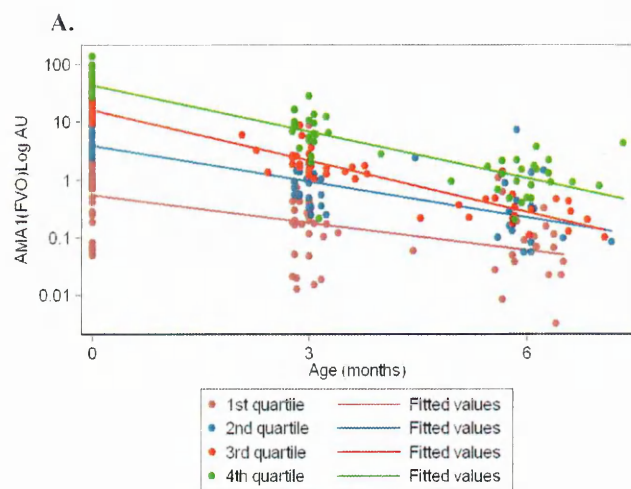
Table 6.4. The rate of decay and antibody half-lives of maternally transferred antibodies to specific antigens tested.

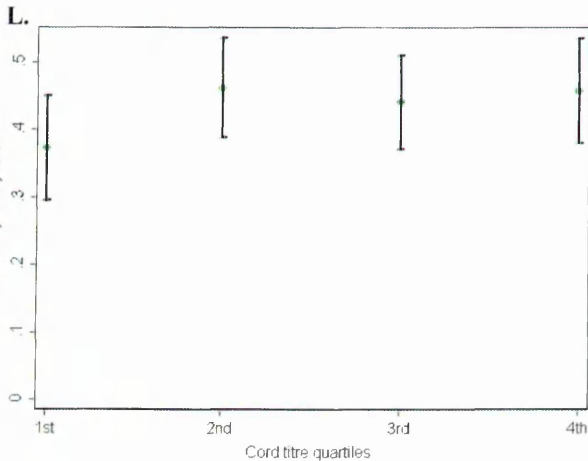
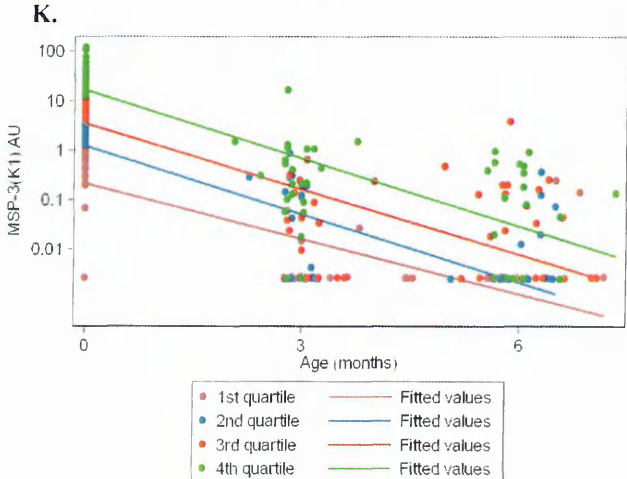
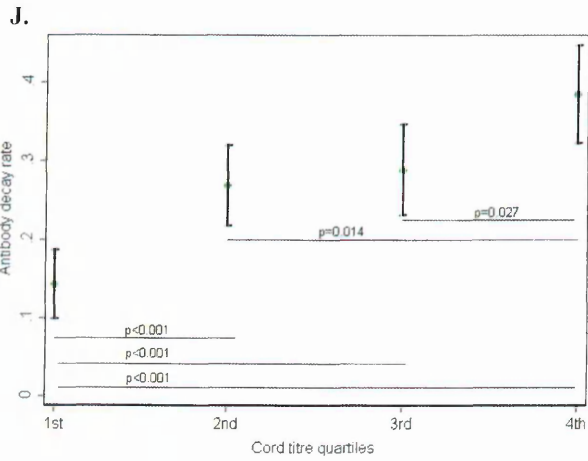
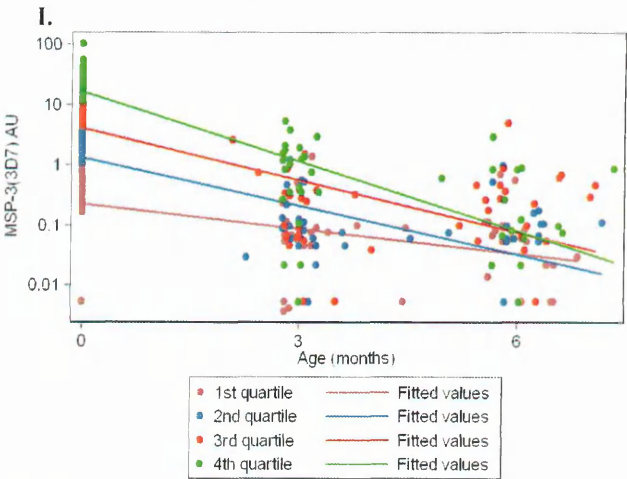
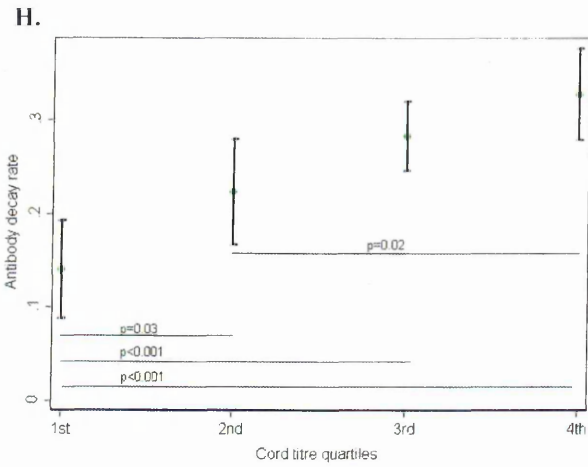
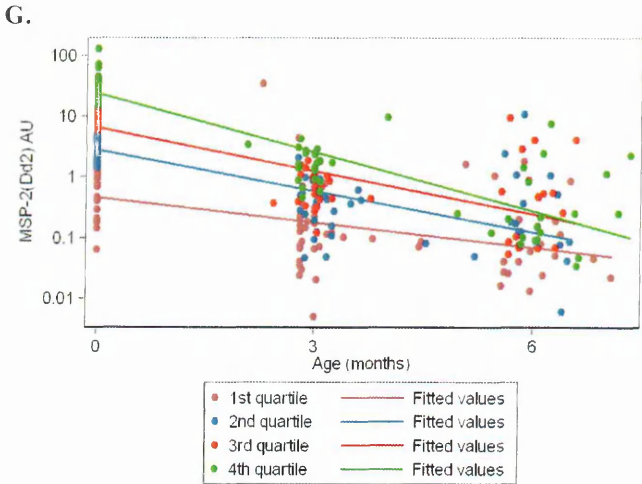
Antigen	Rate of decay* (95% CI)	Antibody half-life in months (95% CI)
AMA1(FVO)	-0.230(-0.248, -0.212)	3.013(2.794 – 3.268)
AMA1(3D7)	-0.222(-0.242, -0.203)	3.122(2.864 – 3.414)
MSP-2(Dd2)	-0.239(-0.263, -0.215)	2.899(2.635 – 3.223)
MSP-2(CH150)	-0.247(-0.272, -0.222)	2.805(2.548 – 3.121)
MSP-3(3D7)	-0.275(-0.308, -0.241)	2.520(2.250 – 2.876)
MSP-3(K1)	-0.451(-0.494, -0.408)	1.537(1.403 – 1.698)
MSP-1 ₁₉	-0.066(-0.093, -0.040)	10.500(7.452 – 17.325)
PfRh2	-0.020(-0.041, -0.0008)	34.650(16.902 – 866.25)

*Log₁₀ reduction per month of indicated values.

6.3.5 Relationship between the rate of decline of cord blood titres and initial titres

Previous studies have shown that the half-life IgG to rubella (Cloonan, Hawkes and Stevens 1970) parainfluenza type 3 and influenza type A2 (Cloonan, Hawkes and Stevens 1971) passively acquired by infants from their mothers is inversely proportional to the initial titre in cord blood. This suggests that the rate of decline of antibodies in infants with high antibody titres is greater than in infants with low level antibodies. To test this finding for malaria-specific antibodies, the rate of decay of specific-antibodies according to different pristine antibody levels was determined. Cord blood titres were divided into quartiles and the decay rate of antibodies within each quartile assessed. With the exception of anti-MSP-3 K1 antibodies (Figure 6.3 L), cord blood titres in the 4th quartile (highest titres) decayed most rapidly followed by those in the 3rd, 2nd and 1st quartiles, in that order (Figure 6.3 – B, D, F, H, J, N and P). For example, the decay rate for MSP-2 Dd2 antibodies was 0.14, 0.22, 0.23 and 0.31 log₁₀ titre reduction per month in the 1st, 2nd, 3rd and 4th cord quartiles, respectively (Figure 6.3 F). Although the general trend was towards a decline in cord blood titres over the 6 months follow-up period, infants with low titres of MSP-1₁₉ and *Pf*Rh2 exhibited a boost in titres as opposed to a decline and hence the negative decay rates shown in Figure 6.3 N and P.





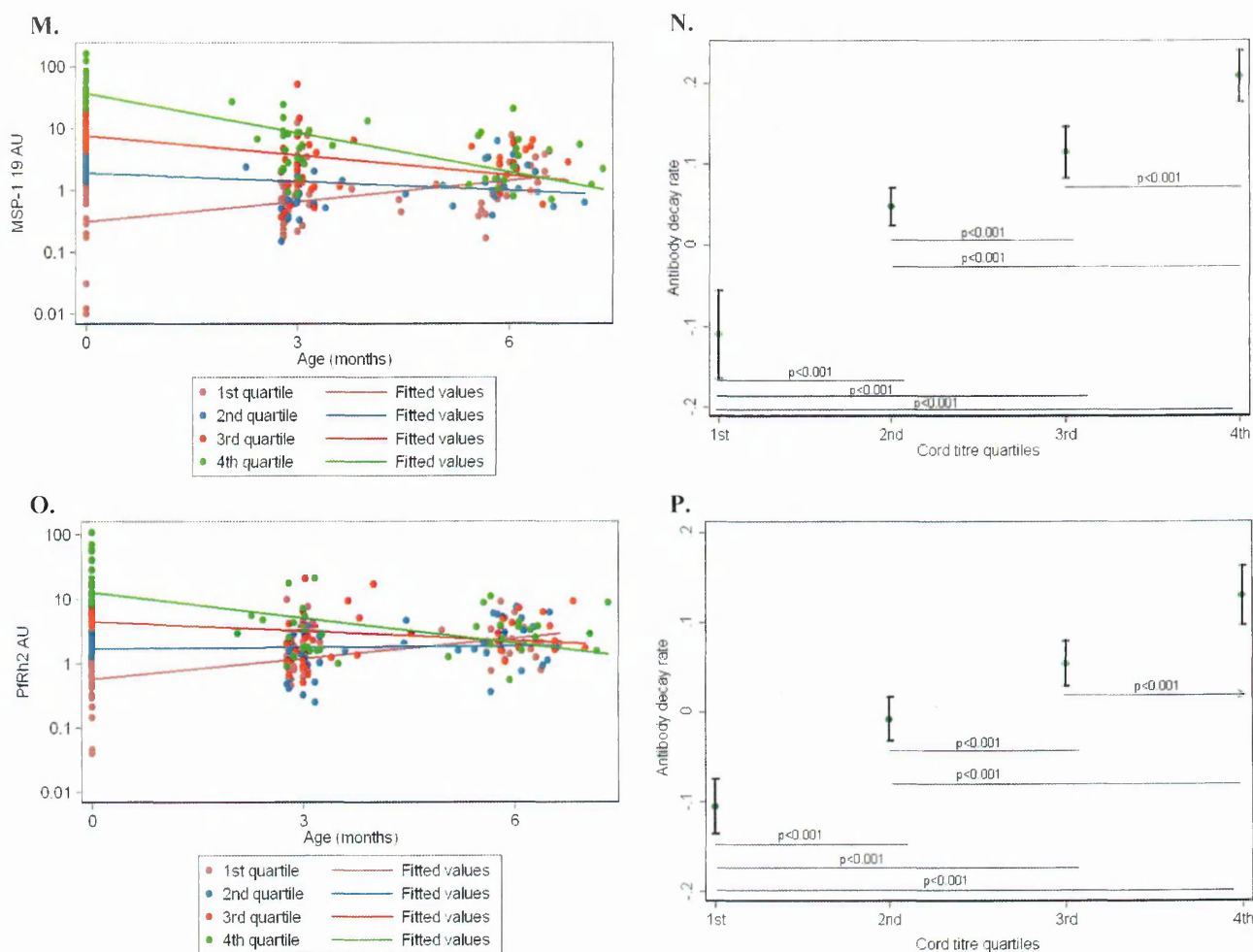
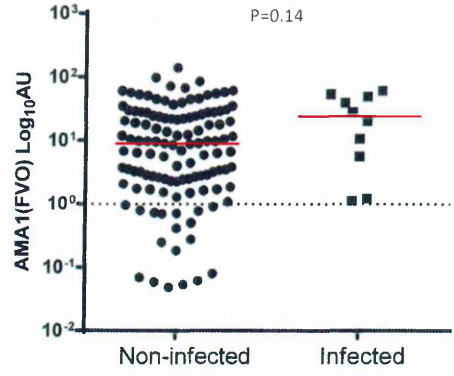


Figure 6.3 Scatter plots of antibody titres to (A) AMA1(FVO), (C) AMA1(3D7), (E) MSP-2(Dd2), (G) MSP-2(CH150/9), (I) MSP-3(3D7), (K) MSP-3(K1), (M) MSP-1₁₉ and (O) *Pf*Rh2, showing the decay rates according to the initial (cord blood) titres. Antibody titres in cord blood were divided into quartiles and regression lines fitted for each quartile as shown in maroon (1st quartile), navy blue (2nd quartile), red (3rd quartile) and green (4th quartile) using data collected during six months of follow-up from birth. The decay rates and 95% CIs of cord blood titres (on the y-axis) according to the different quartile levels (on the x-axis) are shown for the antigens tested (B) AMA1 (FVO), (D) AMA1 (3D7), (F) MSP-2(Dd2), (H) MSP-2(CH150/9), (J) MSP-3(3D7), (L) MSP-3(K1), (N) MSP-1₁₉ and (P) *Pf*Rh2. P values <0.05 indicate differences between regression coefficients (decay rates) that were statistically significant.

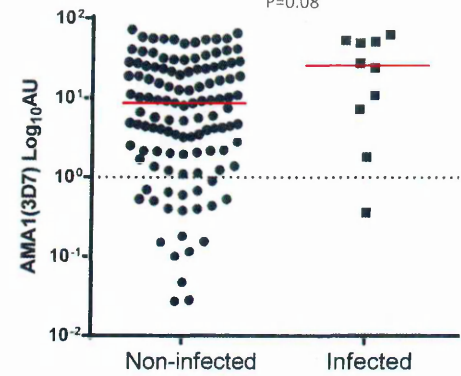
6.3.6 Comparison of maternally transferred antibody titres in children who subsequently acquired asymptomatic parasitemia and those who remained uninfected during the first six months of life

To determine whether susceptibility to asymptomatic infections during the first six months of life could be explained by the levels of maternally transferred antibodies, median cord blood IgG levels against a panel of merozoite antigens were compared in children who acquired infections (N=10) and those who remained uninfected during this period (N=120). There was a trend towards higher median cord blood IgG titres in children who acquired asymptomatic infections compared to those who did not (Figure 6.4.1) and this was of borderline significance for anti MSP-1₁₉ antibodies (Figure 6.4.1G). I also hypothesized that the rate of decay of maternal antibodies after birth could potentially define the duration of protection in infants and therefore compared antibody half-lives for children who acquired asymptomatic infections and those who remained uninfected during the first six months of life. There was no significant difference in median antibody half-lives in the two groups of children for all antigens tested (Figure 6.4.2) suggesting that the longevity of maternal antibodies in these two groups does not influence susceptibility to asymptomatic infection.

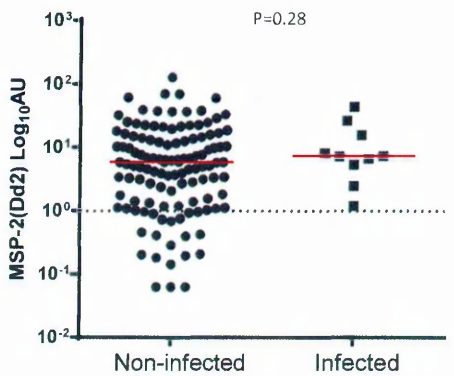
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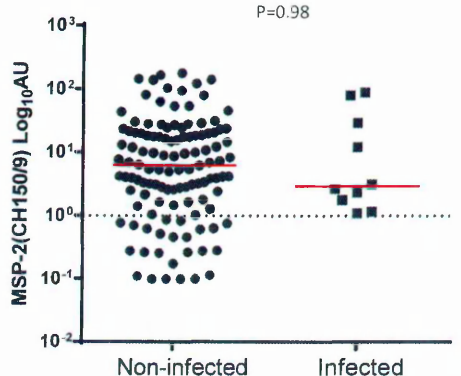
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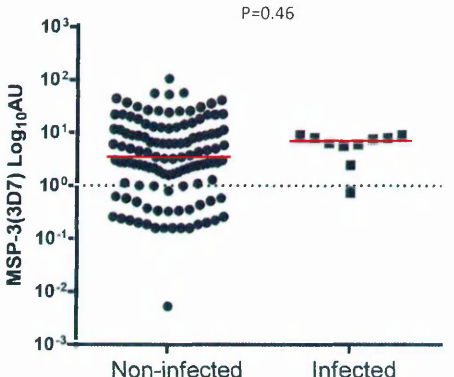
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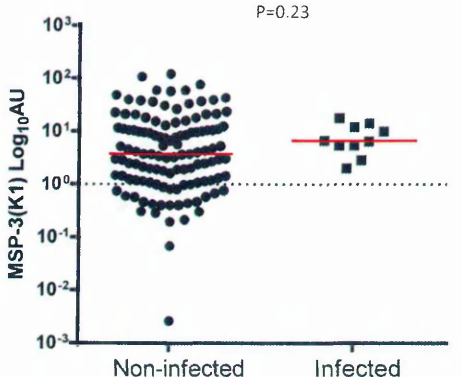
D.



E.



F.



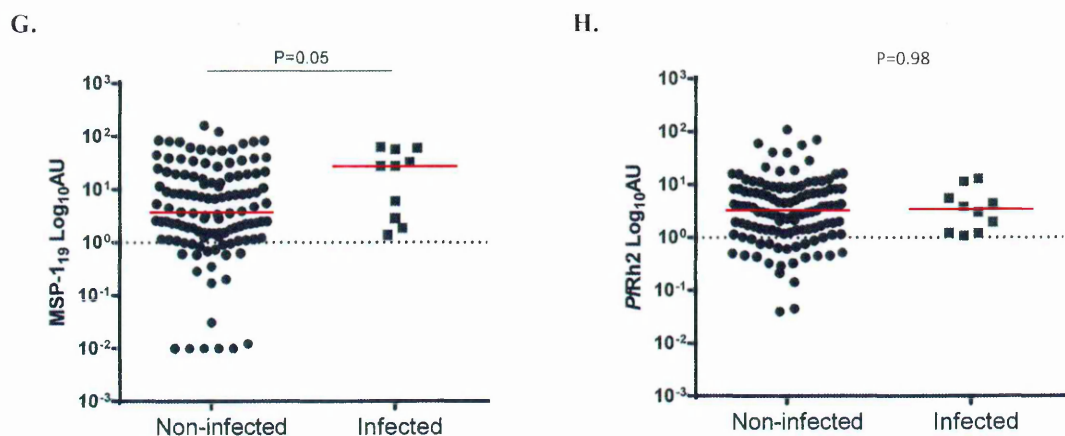
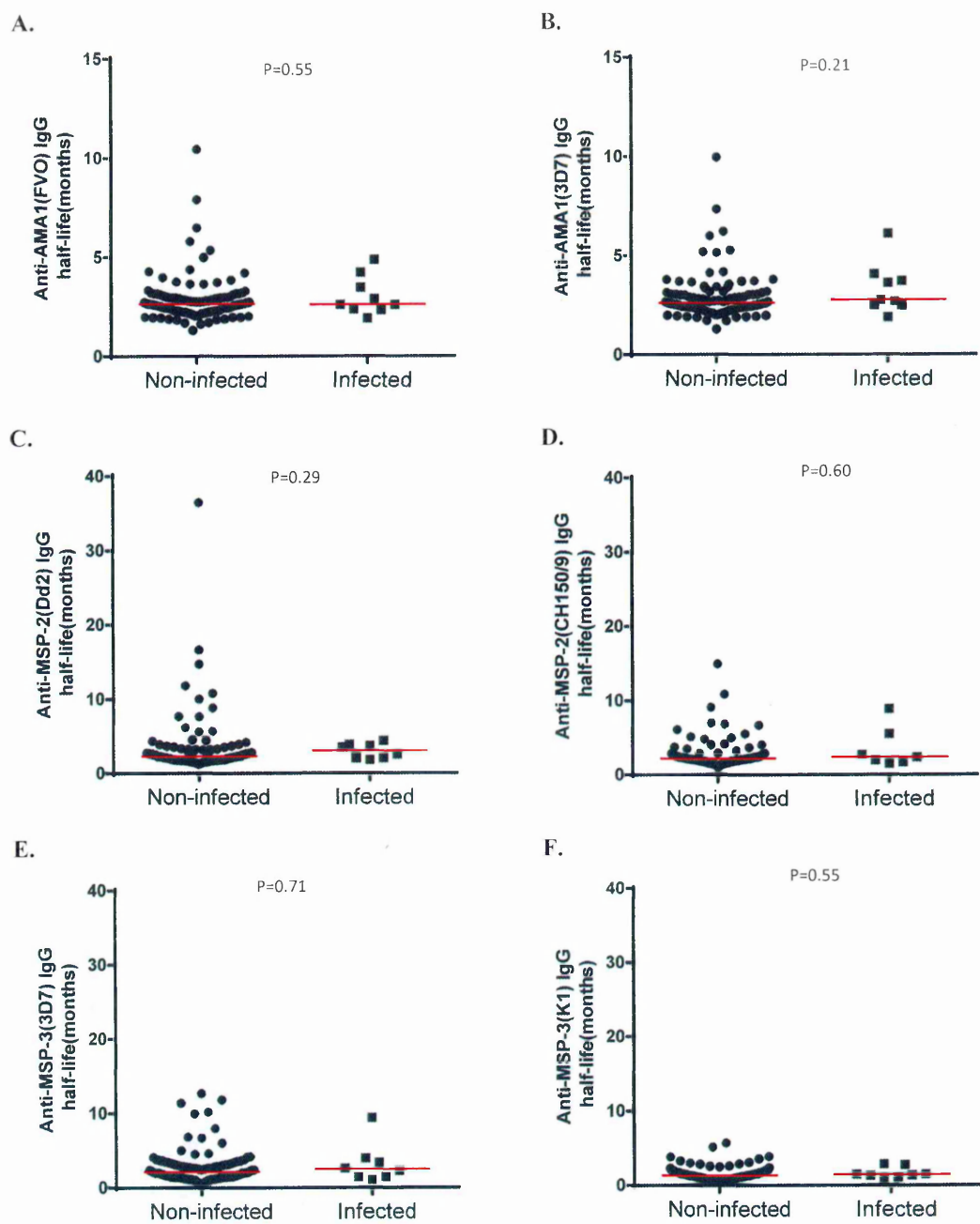


Figure 6.4.1. Cord blood antibody levels against (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *Pf*Rh2 in children who subsequently acquired infections (squares) and those who did not (circles), during six months of follow-up. Horizontal red lines represent the median antibody level for both groups. P values indicating the significance level for comparisons between groups are shown on top of each graph.



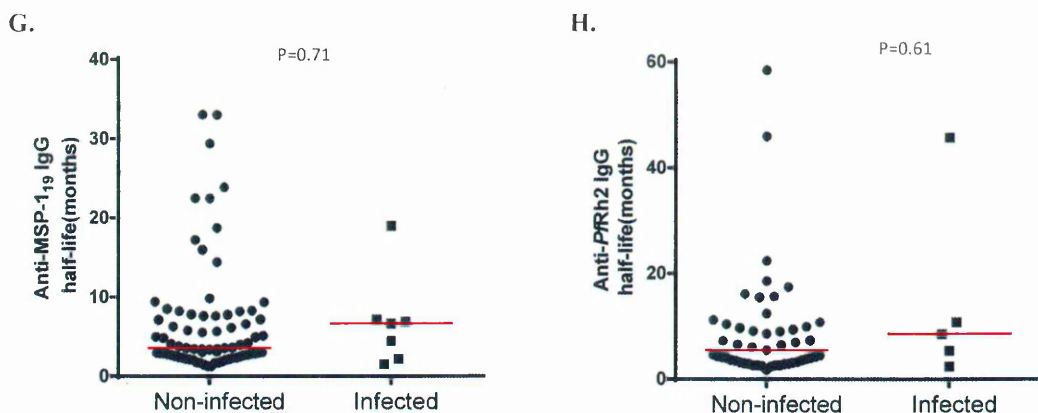
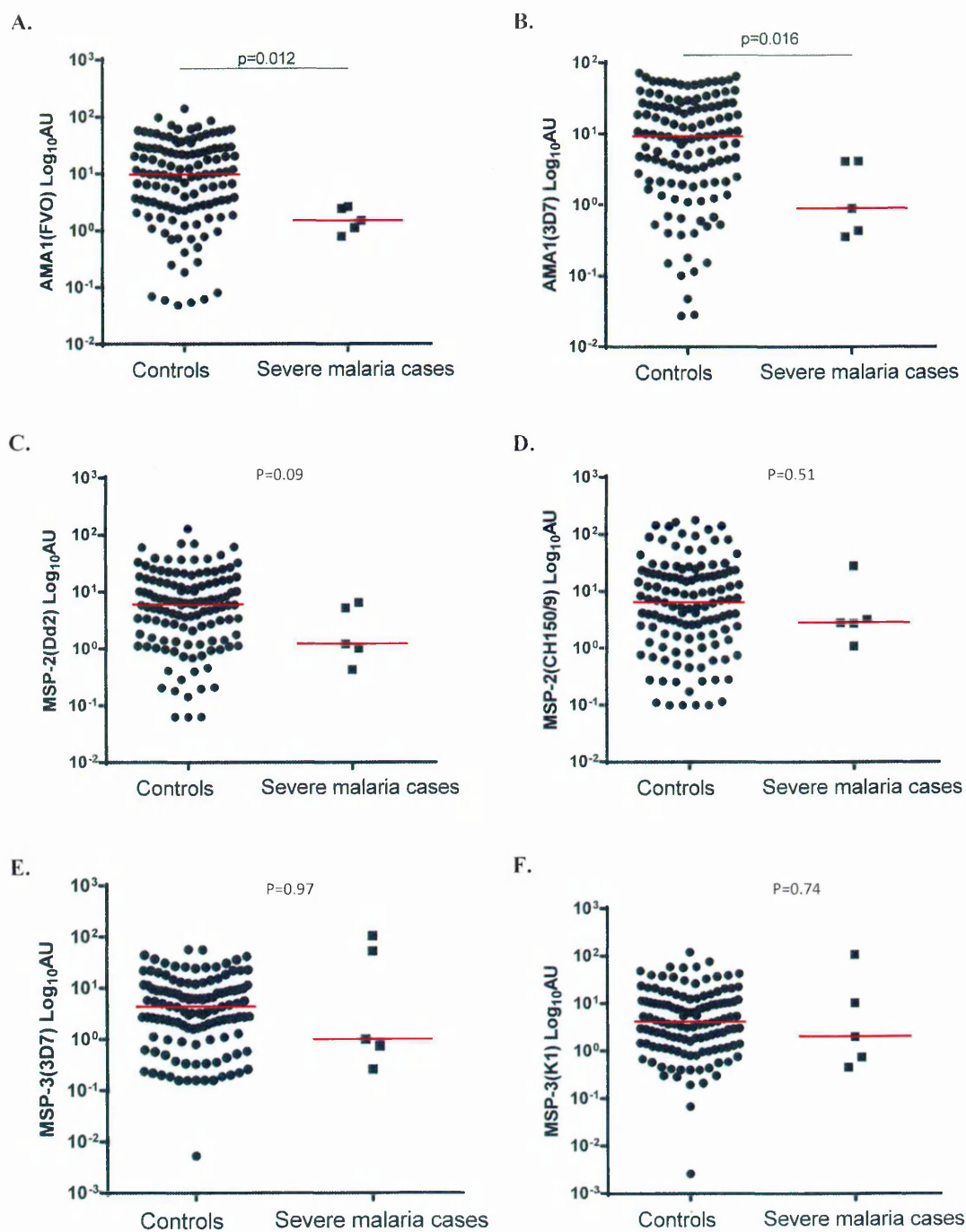


Figure 6.4.2. Scatter plots of the distribution of individual antibody half-lives to (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *Pf*Rh2 in children who subsequently acquired infections (squares) and those who did not (circles) during six months of follow-up. Median antibody half-lives are shown as horizontal red lines. P values indicating the significance level for comparisons between groups are shown on top of each graph.

6.3.7 Comparison of maternally transferred antibody titres in children who subsequently developed severe malaria during the first six months of life and those who did not

For all antigens tested, the median antibody levels in cord blood were lower in children who subsequently developed severe malaria (severe malaria cases) during the first six months of life compared to those who did not (controls) (Figure 6.4.3). The median levels were significantly higher in controls compared to cases for AMA1 alleles ($p=0.012$ and 0.016 for AMA1 (FVO) and AMA1 (3D7) antibodies, respectively) (Figures 6.4.3A and 6.4.3B). In contrast, the median antibody half-lives were significantly higher in severe malaria cases (3.2 months and 3.04 months) compared to controls (2.6 months and 2.6 months) for AMA1 (FVO) and AMA1 (3D7) alleles, respectively (Figures 6.4.4A and 6.4.4B). These results corroborate the earlier findings shown in Figure 6.4.3 that high maternal antibody titres have a shorter half life than relatively lower titres. There was no significant difference in antibody half-lives between cases and controls for the other antigens tested (Figure 6.4.3 C to H).



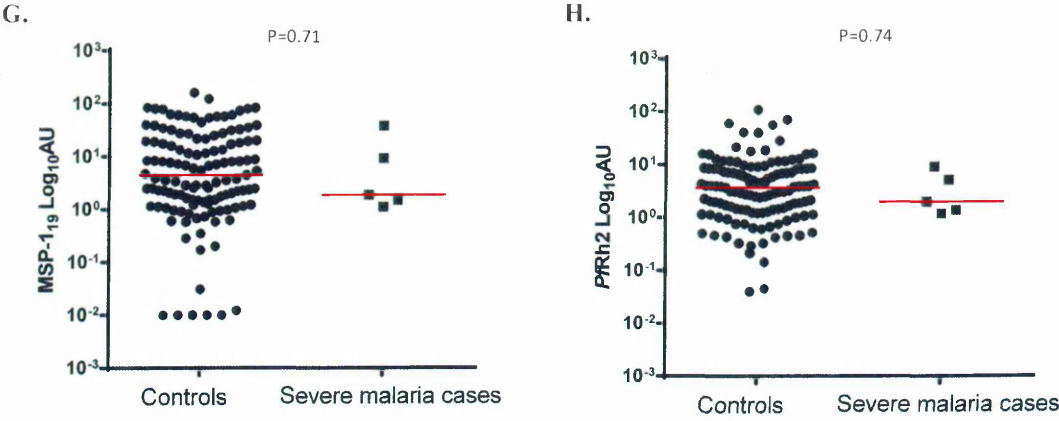
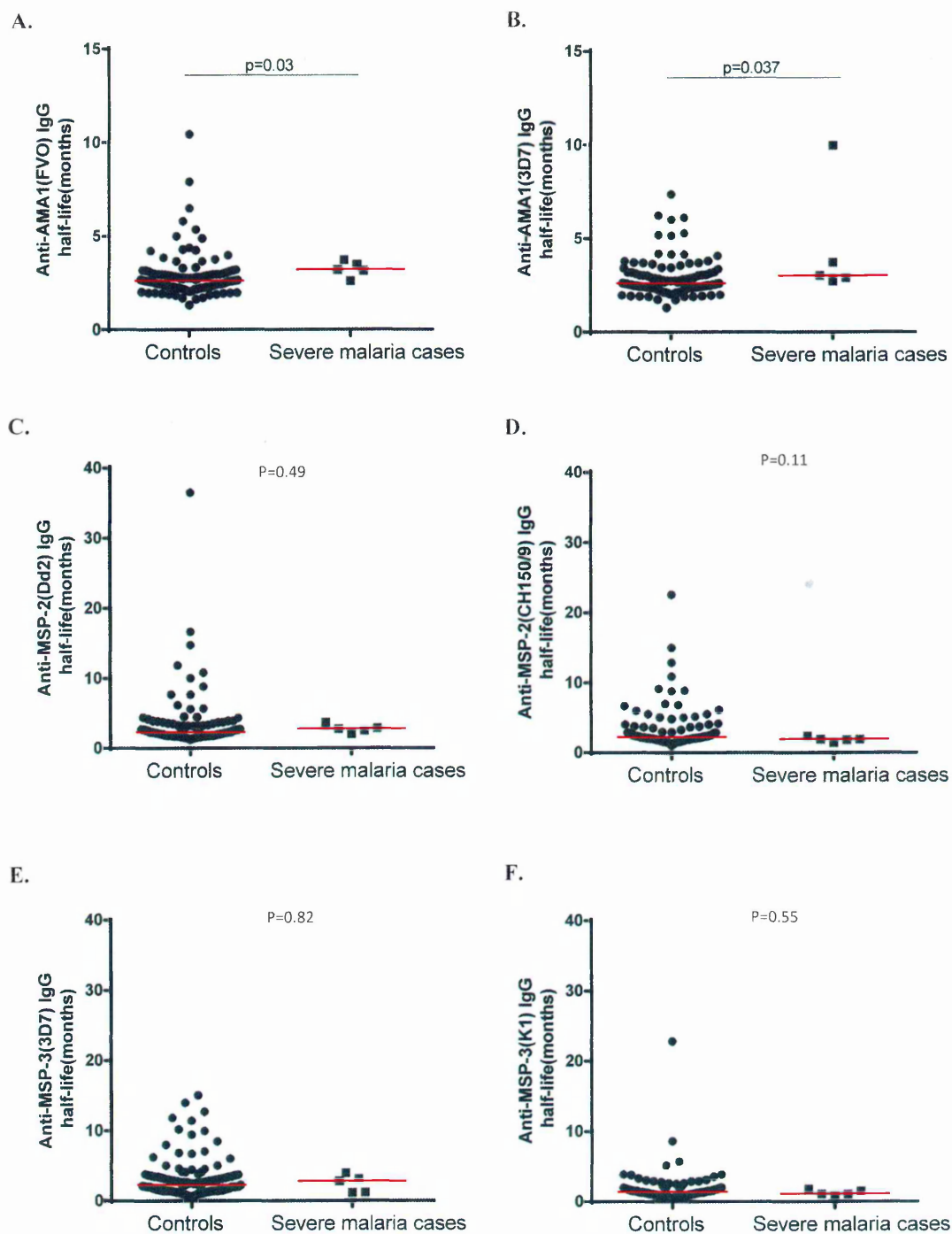


Figure 6.4.3. Cord blood antibody levels against (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) P/Rh2 in children who subsequently developed severe malaria (squares) and those who did not (circles) during six months of follow-up. Horizontal red lines represent the median antibody levels in both groups.



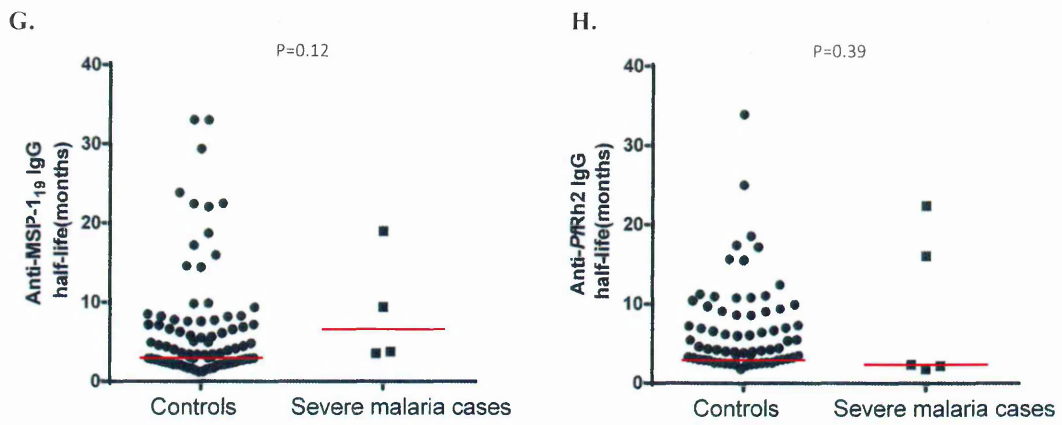


Figure 6.4.4. Scatter plots of the distribution of individual antibody half-lives to (A) AMA1(FVO), (B) AMA1(3D7), (C) MSP-2(Dd2), (D) MSP-2(CH150/9), (E) MSP-3(3D7), (F) MSP-3(K1), (G) MSP-1₁₉ and (H) *Pf*/Rh2 in children who subsequently developed severe malaria (squares) and those who did not (circles) during six months of follow-up. Median antibody half-lives are shown as horizontal red lines.

6.3.8 Maternal antibodies and subsequent risk of acquiring asymptomatic infection or developing severe malaria during the first two years of life.

As demonstrated in chapter 5, merozoite-specific antibodies in cord blood were detectable in a large proportion of infants (94% and 93% for AMA1 FVO and 3D7 respectively, 88% and 85% for MSP-2 Dd2 and CH150/9 respectively, 69% and 66% for MSP-3 3D7 and K1 respectively, 46% for MSP-1₁₉ and 34% for *Pf*Rh2). For this cohort of infants, cord blood titres had a half-life ranging between 1 – 3 months for most antigens tested and I therefore reasoned that these antibodies were likely to be protective only during the first six months of life although other studies have looked at longer periods of follow-up upto 1 year of age (Table 6.1). In order to determine the role of these antibodies in infant protection against asymptomatic infection and severe malaria during the first six months of life and in comparison over the first two years of life, a logistic regression model was used to compare the odds of acquiring an infection during the first six months of life in children who had antibodies and those who did not. For this analysis, two cutoffs were applied: (i) a seropositive cutoff defined by the mean + 3SD of twenty European plasma; and (ii) a threshold cutoff defined in two independent study sites as the dichotomization point that gave the best model fit as defined by the least log likelihood ratio (Murungi et al. 2013, Rono et al. 2013). These levels were 55AU for AMA1, 19AU for MSP-2, 16AU for MSP-3 (3D7) and 59AU for MSP-1₁₉. Threshold levels for *Pf*Rh2 have not yet been defined. The association between transplacentally acquired antibodies and protection against infection during the first two years of life was also evaluated.

Based on the cutoff of seropositivity, none of the children who were seronegative for AMA1 and MSP-2 alleles acquired asymptomatic infections during the follow-up period compared to 8-9% who were seropositive but subsequently acquired an infection (Table 6.5.1). Similarly, for the remaining antigens, presence of specific-antibodies was associated with an increased

risk of acquiring an infection during the first six months of life in multivariate analysis (OR 4.54(0.53-38.58, $p=0.16$, OR 5.27(0.62-44.34) $p=0.12$ and OR 1.94(0.47-7.96), $p=0.35$ for MSP-3(3D7), MSP-3(K1) and MSP-1₁₉), respectively (Table 6.5.1). Assessment of the role of these antibodies in protection against infection during the first two years of life also revealed an increased risk of acquiring infections in children who were seropositive compared to those who were seronegative (OR 1.24(0.14-10.96), $p=0.84$, OR 1.46(0.16-12.65), $p=0.73$, OR 3.49(0.95-12.86), OR 4.25(1.16-15.50), $p=0.02$ and OR 1.38(0.53-3.58), $p=0.50$ for AMA1(FVO), AMA1(3D7), MSP-3(3D7), MSP-3(K1) and MSP-1₁₉ respectively (Table 6.5.2). These results suggest that antibodies present in cord blood are a marker of exposure in this group of children although the estimates were not statistically significant. There was a trend towards a reduced risk of acquiring an asymptomatic infection in children who had antibodies to *Pf*Rh2 during the first six months or two years of follow-up (OR 0.42(0.08-2.14), $p=0.30$ and OR 0.44(0.15-1.24), $p=0.12$), respectively, but the estimates were not statistically significant.

The level of antibodies present in cord blood plasma were above the proposed thresholds associated with protection against clinical episodes of malaria in 11% and 8% of infants in this birth cohort for AMA1 (FVO and 3D7, respectively), 25% and 35% for MSP-2 (Dd2 and CH150/9, respectively) and 23% and 25% for MSP-3(3D7 and K1, respectively). However, these antibodies were associated with a non-significant increase in susceptibility to asymptomatic infections suggesting that they are markers of exposure to infection (OR 1.19(0.13-10.86), $p=0.87$, OR 1.78(0.19-16.12), $p=0.60$, OR 1.03(0.19-5.38), $p=0.97$, OR 1.17(0.24-5.57), $p=0.84$ and OR 3.56(0.55-22.76), $p=0.17$ for AMA1 (FVO), AMA1(3D7), MSP-2(Dd2), MSP-2(CH150/9) and MSP-1₁₉, respectively). There was a trend towards reduced risk in children who had antibodies above the threshold level to MSP-3 (K1), OR 0.39(0.04-3.57), $p=0.40$ but the estimates were not significant (Figure 6.5.1). In analyses of

two years of follow-up from birth, presence of antibodies above the threshold was associated with increased risk of acquiring an infection for all antigens tested (OR 1.88(0.47-7.47), $p=0.36$, OR 1.34(0.25-7.18), $p=0.72$, OR 1.73(0.63-4.77), $p=0.28$, OR 2.62(0.99-7.02), $p=0.84$, OR 0.96(0.30-3.06), $p=0.94$, OR 1.52(0.52-4.39), $p=0.43$ and OR 0.77(0.14-3.99) $p=0.75$ for AMA1 (FVO), AMA1 (3D7), MSP-2(Dd2), MSP-2(CH150/9), MSP-3(3D7), MSP-3(K1), *Pf*Rh2 and MSP-1₁₉, respectively (Table 6.5.2).

Table 6.5.1. The protective effect of specific-antibodies against acquisition of asymptomatic parasitemia during the first six months of life.
Analyses are based on antibody seropositivity and protective thresholds.

Seropositivity cutoffs					Threshold cutoffs			
	IgG positive and got infected %(n/N)	IgG negative and got infected %(n/N)	Univariate analysis	Multivariate analysis ¹	IgG positive and got infected %(n/N)	IgG negative and got infected %(n/N)	Univariate analysis	Multivariate analysis ¹
AMAI(FVO)	8.1% (10/123)	0% (0/7)	ND ¹	ND	9.1%(1/11)	7.5%(9/119)	1.22(0.14-10.65)	1.19(0.13- 10.86)
AMAI(3D7)	8.2% (10/122)	0% (0/8)	ND	ND	12.5%(1/8)	7.3%(9/122)	1.79(0.19-16.22)	1.78(0.19-16.12)
MSP-2(Dd2)	8.7% (10/115)	0% (0/15)	ND	ND	8.0%(2/25)	7.6%(8/105)	1.05(0.20-5.30)	1.03(0.19-5.38)
MSP-2(CH150)	9.0%(10/111)	0% (0/19)	ND	ND	8.5%(3/35)	7.3%(7/95)	1.17(0.28-4.83)	1.17(0.24-5.57)
MSP-3(3D7)	10.0% (9/90)	2.5% (1/40)	4.33(0.53-35.42)	4.54(0.53-38.58)	0%(0/23)	9.3%(10/107)	ND	ND
MSP-3(K1)	10.3% (9/87)	2.3% (1/43)	4.84(0.59-39.56)	5.27(0.62-44.34)	4.0%(1/25)	8.5%(9/105)	0.44(0.05-3.68)	0.39(0.04-3.57)
PfPRh2	4.4% (2/45)	9.4% (8/85)	0.44(0.09-2.20)	0.42(0.08-2.14)	ND ²	ND	ND	ND
MSP-I ₁₉	10.0% (6/60)	5.7% (4/70)	1.83(0.49-6.83)	1.94(0.47-7.96)	18.1%(2/11)	6.7%(8/119)	3.08(0.56-16.74)	3.56(0.55-22.76)
			0.36	0.35			0.19	0.17

¹In multivariate analyses, adjustment for the confounding effect of cumulative exposure to infection (as measured by antibody reactivity

to schizont extract (fitted as continuous covariates)) was included in the logistic model.

*ND. Not determined.

Table 6.5.2. The protective effect of specific-antibodies against acquisition of asymptomatic parasitemia during the first two years of life. Analyses are based on antibody seropositivity and protective threshold cutoffs.

Seropositivity cutoffs					Threshold cutoffs		
	IgG positive and got infected %(n/N)	IgG negative and got infected %(n/N)	Univariate analysis ¹	Multivariate analysis ¹	IgG positive and got infected %(n/N)	IgG negative and got infected %(n/N)	Multivariate analysis ¹
AMA1(FVO)	21% (26/123)	14% (1/7)	1.60(0.18-13.95)	1.24(0.14-10.96)	36% (4/11)	19% (23/119)	1.88(0.47-7.47)
AMA1(3D7)	21% (26/122)	12% (1/8)	1.89(0.22-16.10)	1.46(0.16-12.65)	25% (2/8)	20% (25/122)	1.34(0.25-7.18)
MSP-2(Dd2)	23% (27/115)	0% (0/15)	ND	ND	32% (8/25)	18% (19/105)	1.73(0.63-4.77)
MSP-2(CH150)	24% (27/111)	0% (0/19)	ND	ND	37% (13/35)	14% (14/95)	2.62(1.00-7.02)
MSP-3(3D7)	26% (24/90)	7% (3/40)	4.48(1.26-15.90)	3.49(0.95-12.86)	26% (6/23)	19% (21/107)	0.96(0.30-3.06)
MSP-3(K1)	27% (24/87)	6% (3/43)	5.07(1.43-17.97)	4.25(1.16-15.50)	32% (8/25)	18% (19/105)	1.52(0.52-4.39)
PfPRh2	15% (7/45)	23% (20/85)	0.59(0.23-1.54)	0.44(0.15-1.24)	ND	ND	ND
MSP-1 ₁₉	26% (16/60)	15% (11/70)	1.95(0.82-4.61)	1.38(0.53-3.58)	27% (3/11)	20% (24/119)	0.77(0.14-3.99)
			0.12	0.50			0.75

¹In multivariate analyses, adjustment for the confounding effect of cumulative exposure to infection (as measured by antibody reactivity to schizont extract (fitted as a continuous covariate) was included in the logistic model.

*ND. Not determined.

6.3.9 Maternal antibodies and subsequent risk of developing severe malaria during the first two years of life.

Further analysis of infant protection by maternal antibodies during the first six months of life revealed a non-significant reduction in risk of developing severe malaria in children who had antibodies above the seropositivity cutoff to MSP-3(3D7), MSP-3(K1) and *Pf*Rh2 (OR 0.52 (0.07-3.71), $p=0.52$, OR 0.58 (0.08-3.96), $p=0.58$ and 0.61 (0.06-5.82), $p=0.67$; respectively) (Table 6.6.1). Similarly, over a longer period of follow-up (up to 2 years), antibodies to AMA1, MSP-3 and MSP-1₁₉ were also associated with a reduction in risk of developing severe malaria but the estimates were not statistically significant (Table 6.6.2). None of the children that had antibodies above the threshold levels to AMA1 alleles and MSP-2(Dd2) developed severe malaria (Table 6.6.1). Children who had cord blood titres above the threshold cutoffs for MSP-2(CH150/9), MSP-3(3D7) and MSP-3(K1) had an increased susceptibility of developing severe malaria during the first six months of life suggesting that these high antibody titres were acting as markers that could identify children who had higher exposure to *Pf* infections and were therefore also more likely to develop severe malaria, (OR 2.07 (0.19-22.61), $p=0.55$, OR 15.00 (1.63-37.96), $p=0.017$, OR 3.00 (0.27-32.58), $p=0.36$), respectively (Table 6.6.1). Similar findings were also observed when children were followed up to 2 years of age (Table 6.6.2)

Table 6.6.1. The protective effect of specific-antibodies against development of severe malaria during the first six months of life. Analyses are based on antibody seropositivity and protective thresholds.

Seropositivity cutoffs					Threshold cutoffs			
	IgG positive and developed SM %(n/N)	IgG negative and developed SM %(n/N)	Univariate analysis	Multivariate analysis ¹	IgG positive and developed SM %(n/N)	IgG negative and developed SM %(n/N)	Univariate analysis	Multivariate analysis ¹
AMAI(FVO)	4.0%(5/123)	0% (0/7)	ND*	ND	0%(0/11)	4.2%(5/119)	ND	ND
AMAI(3D7)	4.1%(5/122)	0% (0/8)	ND	ND	0%(0/8)	4.1%(5/122)	ND	ND
MSP-2(Dd2)	3.4%(4/115)	6.6% (1/15)	0.50(0.05-4.83)	1.01(0.09-10.52)	0%(0/25)	4.7%(5/105)	ND	ND
MSP-2(CH150)	4.5%(5/111)	0% (0/19)	ND	ND	4.2%(4/95)	2.8%(1/35)	0.66(0.07- 6.20)	2.07(0.19-22.61)
MSP-3(3D7)	2.2%(2/90)	7.5% (3/40)	0.28(0.04-1.74)	0.52(0.07-3.71)	8.7%(2/23)	2.8%(3/107)	3.30(0.51- 20.98)	15.00(1.63-37.96)
MSP-3(K1)	2.3%(2/87)	6.9% (3/43)	0.31(0.05-1.95)	0.58(0.08-3.96)	4.0%(1/25)	3.8%(4/105)	1.05(0.11- 9.84)	3.00(0.27-32.58)
PfRh2	2.2%(1/45)	4.7% (4/85)	0.46(0.04-4.24)	0.61(0.06-5.82)	N/A	N/A	N/A	N/A
MSP-1 ₁₉	3.3%(2/60)	4.2% (3/70)	0.77(0.12-4.76)	1.31(0.31-17.26)	0%(0/11)	4.2%(5/119)	ND	ND

¹In multivariate analyses, adjustment for the confounding effect of cumulative exposure to infection (as measured by antibody reactivity to schizont extract (fitted as a continuous covariate) was included in the logistic model.

*ND. Not determined.

Table 6.6.2. The protective effect of specific-antibodies against development of severe malaria during the first two years of life.

Analyses are based on antibody seropositivity and protective threshold cutoffs.

Seropositivity cutoffs					Threshold cutoffs			
	IgG positive and developed SM %(n/N)	IgG negative and developed SM %(n/N)	Univariate analysis	Multivariate analysis ¹	IgG positive and developed SM %(n/N)	IgG negative and developed SM %(n/N)	Univariate analysis	Multivariate analysis ¹
AMAI(FVO)	20%(23/115)	28% (2/7)	0.62(0.10-3.42)	0.64(0.11-3.59)	20%(2/10)	20%(23/112)	0.96(0.19-4.86)	0.98(0.19-5.12)
AMAI(3D7)	20%(23/114)	25% (1/8)	0.75(0.14-4.00)	0.78(0.14-4.22)	14%(1/7)	20%(24/115)	0.63(0.07-5.50)	0.64(0.07-5.44)
MSP-2(Dd2)	20%(22/107)	20% (3/15)	1.03(0.26-3.99)	1.08(0.27-4.26)	18%(4/22)	21%(21/100)	0.83(0.25-2.73)	0.84(0.24-2.85)
MSP-2(CH150)	22%(23/103)	10% (2/19)	2.44(0.52-11.36)	2.64(0.55-12.56)	21%(7/32)	20%(18/90)	1.12(0.41- 2.99)	1.19(0.40-3.56)
MSP-3(3D7)	19%(16/83)	23% (9/39)	0.79(0.31-2.00)	0.77(0.28-2.06)	22%(5/22)	20%(20/100)	1.17(0.38- 3.57)	1.23(0.38-3.96)
MSP-3(K1)	18%(15/80)	23% (10/42)	0.73(0.29-1.82)	0.75(0.28-1.95)	25%(6/24)	19%(4/105)	1.38(0.48-3.96)	1.49(0.48-4.58)
PfPRh2	21%(9/42)	20% (16/80)	1.09(0.43-2.73)	1.09(0.43-2.79)	N/A	N/A	N/A	N/A
MSP-I ₁₉	18%(10/55)	22% (15/67)	0.77(0.31-1.88)	0.74(0.27-2.00)	40%(4/10)	18%(21/112)	2.88(0.74-11.15)	3.77(0.82-17.22)
			0.56	0.56			0.12	0.08

¹In multivariate analyses, adjustment for the confounding effect of cumulative exposure to infection (as measured by antibody reactivity to schizont extract (fitted as a continuous covariate) was included in the logistic model.

238

*ND. Not determined.

6.3.10 Relationship between breadth of responses and protection against asymptomatic infection and severe malaria during the first six months of life

The breadth of anti-merozoite responses present in cord blood plasma was compared between: i) infants who subsequently got asymptomatic infections during the first six months of life and those who did not (Figure 6.5 A); and ii) infants who subsequently developed severe malaria during the first six months of life and those who did not (Figure 6.5 B). Cord blood plasma from a large proportion of children who acquired asymptomatic infections (90%) recognized three or more antigens compared to children who did not acquire asymptomatic infections (75%) suggesting that antibody breadth was a marker of exposure in this group of infants. Also noted was that all the children who acquired asymptomatic infections had responses to at least two antigens or more (Figure 6.5 A). In contrast, only 40% of the children who developed severe malaria recognized three or more antigens compared to 77% of the children who did not develop severe malaria suggesting that antibody breadth could be protective against development of severe malaria (Figure 6.5 B). It was not possible to determine the protective efficacy according to different levels of antibody breadth due to the very small numbers obtained in the sub group analysis.

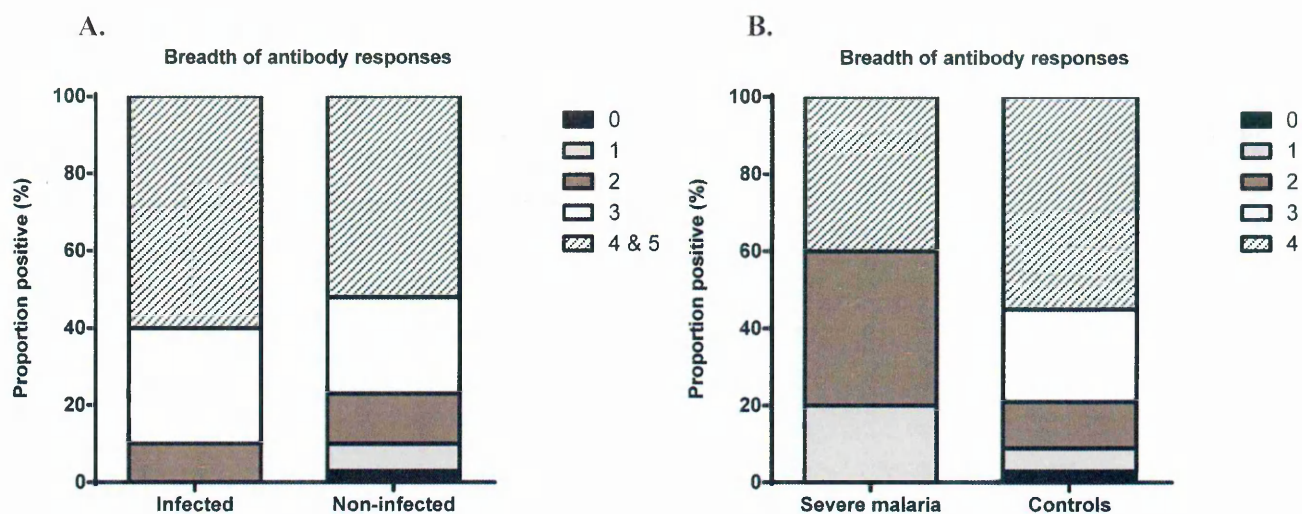


Figure 6.5 The breadth of anti-merozoite antibody responses in cord blood plasma samples from children who acquired asymptomatic infections during the first six months of life versus those who did (A) and from children who developed severe malaria during the first six months of life versus those who remained disease free (B). A total of five antigens (AMA1, MSP-2, MSP-3, *Pf*Rh2 and MSP-1₁₉) were tested. Black, light grey, dark grey, white and shaded bars represent the proportion of individuals responding to 0, 1, 2, 3 and >4 antigens, respectively.

6.4 Discussion

In this chapter, I have evaluated the role of the concentration of transplacentally acquired antibodies against merozoite antigens in protection against acquisition of infection and development of severe malaria during the first six months of life and also extended the analysis to a follow up of up to two years of age. The analyses are based on a limited sample set of one hundred and thirty cord blood plasma samples and a very small proportion of children acquiring infections or developing severe malaria during the first six months of

follow up (7.6% and 4.1%, respectively) therefore lacking adequate statistical power to detect significant differences and at best only able to infer trends from the data.

The prevalence of transplacentally acquired antibodies against merozoite antigens in this cohort of infants was high and comparable to what has previously been reported for AMA1, and MSP-2 (Riley et al. 2000) but much lower for MSP-1₁₉ compared to other studies (Hogh et al. 1995, Kitua et al. 1999), suggesting that MSP-1₁₉ is not very immunogenic in this population. Infants who were seropositive for the panel of antigens tested had an increased risk of acquiring asymptomatic infections during the first six months of life, a period in which maternal antibodies are more likely to persist and also when the analysis was extended to a longer period of follow-up of 2 years of age. Similarly, a greater proportion of children who subsequently got infected had responses to a broader range of the merozoite targets tested. These results therefore suggest that maternally acquired anti-merozoite antibodies are markers of exposure to infection and predict the likelihood of acquiring asymptomatic infections in the first two years of life. These findings are consistent with most studies showing lack of protection by maternal antibodies against malaria antigens in children followed up for up to 6 months (Riley et al. 2000, Riley et al. 2001) or one year of age (Achidi et al. 1996, Hogh et al. 1995, Kitua et al. 1999). In contrast, only one study has demonstrated a protective role for maternal MSP-1₁₉ IgG in prolonging the time to first infection in infants followed up for up to one year of age (Branch et al. 1998). Although I did not detect any differences in directionality of odds ratio estimates between analyses conducted when maternally derived antibodies would be expected to play a protective role (6 months of life) and when extended beyond this period (2 years of age), I would argue that studies should take into account the precise duration over which these antibodies are likely to persist in the infant. However, thus far, there has been a paucity of such data and I have, to the best of my knowledge, provided the first description of half-life estimates of maternally transferred anti-merozoite IgG. Furthermore, regardless of the few previous descriptions of the short duration of maternal antibodies in terms of the time it

takes for the levels to fall to a minimum level, which ranges across studies between 2 months (Kitua et al. 1999), 14 weeks (Riley et al. 2000) and 4-6 months (Achidi et al. 1996), many studies have continued to assess the protective efficacy of maternal antibodies for up to 1 year of age (Kitua et al. 1999, Branch et al. 1998, Hogg et al. 1995).

I have previously described threshold concentrations of anti-merozoite antibodies associated with a reduced risk of developing clinical episodes of malaria (Murungi et al. 2013, Rono et al. 2013). Although these thresholds were specifically defined for clinical episodes of malaria as an endpoint of interest (and were not defined with maternally acquired antibodies but a child's own naturally-acquired antibodies), I explored whether these exact cutoffs as measured in cord blood could also be important for protection against infection and development of severe malaria in infants. However, I found no evidence of their role in protection against infection and severe malaria. From these findings, it is plausible that the threshold levels against the different outcomes of an infection are different but this remains to be demonstrated in a bigger study with adequate cases of severe malaria and an adequate number of individuals with asymptomatic infections.

I have also demonstrated, to my knowledge, the first investigation of the role of these antibodies in protection against severe malaria during the first six months of life and also extended the analysis to two years of age and showed that maternal antibodies against MSP-3 alleles and *PfRh2* conferred a reduced odds of developing severe malaria, whereas AMA1 and MSP-2 antibodies were associated with an increased odds. However, the estimates were not statistically significant and the odds of developing severe malaria in children aged less than six months in this population was very low (3.8%), as previously described in an early study (Snow et al. 1998). When the analysis was extended to two years of follow-up, AMA1, MSP-3(3D7) and *PfRh2* antibodies showed a trend towards reduced odds of developing severe malaria. These findings, together with the observation that children who developed severe malaria generally had lower cord blood antibody levels and also had a narrower breath of

responses compared to children who did not develop severe malaria, suggest that these antibodies could be protective but the very small numbers in this study precluded a conclusive analysis of their protective efficacy.

The kinetics of decay of maternal antibodies to the antigens tested was also examined in detail and was generally shown to decline to half the original concentration within 1.5 months to 3 months to most antigens tested with the exception of anti-MSP-1₁₉ and *Pf*Rh2 antibodies which declined at a much slower rate, up to 34 months. Antibodies to the different antigens declined to a median of 10% - 1% of initial levels by six months of age. Although previous studies have not described the duration of persistence of maternal in terms of the antibody half-life or rates of decay, my findings are consistent with what was shown, that maternal antibodies have a median duration of 14 weeks (range 6-22) (Riley et al. 2000). Moreover, the antibodies fall to minimal levels by 4 months to 10 months of age (Achidi et al. 1996, Sehgal, Siddjiqui and Alpers 1989, Riley et al. 2001), although one study showed a much shorter duration of persistence of two months (Kitua et al. 1999). I also observed that antibody decay rates were inversely proportional to the initial titres present in cord blood, a finding which was in contrast to a study by Riley *et al.*, showing that cord blood antibody titres in Ghanaian infants persisted for a long duration in infants who had high antibody levels at birth (Riley et al. 2000). However, other studies have shown that maternally transferred antibodies to viral antigens, declined rapidly in children with high titres at birth (Cloonan et al. 1970, Cloonan et al. 1971) suggesting that children with moderate to low initial antibody titres would be protected for a longer duration in comparison to those with high antibody titres at birth. Similarly, following acute malaria infection in children, merozoite-specific IgG subclasses were boosted to high levels and displayed a more rapid decline than the theoretical half-life given for these antibodies (Kinyanjui et al. 2007). These data have important implications for malaria vaccines administered in infants. If these vaccines potentially protect by inducing high

titre antibodies and yet are expected to wane very rapidly following immunization, they would require constant boosting during infancy to maintain their levels.

Other factors that may influence the levels of transplacentally acquired merozoite-specific antibodies were also investigated and, similar to a previous observation (Achidi et al. 1996), antibody concentrations measured in cord blood were not associated with parity, maternal age, birth weight or residential location but with previous cumulative exposure to infection by the mother. In contrast, other studies have shown that parity (Mayor et al. 2011, Ricke et al. 2000) and placental infection (Mayor et al. 2011) are associated with increased levels of total IgG to CSA and merozoite antigens, respectively. The unavailability of data on placental infection, intermittent preventive treatment during pregnancy, use of insecticide treated nets and gestational age at delivery was a major limitation of my study. These factors have been shown to influence levels of malaria-specific antibodies in pregnancy, which in turn contributes to the titres transferred from the mother to the infant.

In summary these data show that maternally transferred antibody concentrations do not account for the immunity against severe malaria and asymptomatic infections observed during the first six months of life. Further investigation to; i) determine the role of other factors such as HbF present in young infants, acting in cooperation with cord blood antibodies that could mediate protection; ii) determine the role of other antibody specificities such as IgG subclasses and antibody function and; iii) determine the role of less well-studied antigens in protection, could potentially contribute to our understanding of the mechanisms associated with immunity to malaria early in life.

CHAPTER SEVEN

The role of antibody-mediated effector functions in protection against severe malaria

7.0 Introduction

Functional immunoassays are important tools used to prioritize antigens for development and testing in vaccine trials. For the well-studied merozoite antigens, antibodies that may be important in clearing parasites (Cohen et al. 1961, Sabchareon et al. 1991) have been shown to act by: i) blocking merozoite dispersal after schizont rupture (Green et al. 1981); ii) blocking merozoite invasion into red blood cells (Blackman et al. 1990, Kocken et al. 2002); or iii) inhibiting replication within the infected red blood cell (Locher et al. 1996) in *in vitro* experiments. The relative contribution of these mechanisms to clinical protection in human studies is however less clear.

In vitro invasion assays and growth inhibition activity assays (IIA and GIA) are widely used to measure the ability of antibodies induced following vaccination or those acquired naturally, to block invasion and limit multiplication of laboratory adapted isolates or transgenic parasite lines expressing specific merozoite antigens. Interestingly, GIA is detectable in children aged less than five years of age and either declines or remains stable with increasing age (Duncan, Hill and Ellis 2012, Marsh et al. 1989, Dent et al. 2008, McCallum et al. 2008, Bejon et al. 2011a, Courtin et al. 2009) which is paradoxical to the observed age-related increase in antibody titres to most blood-stage antigens. The studies that have measured GIA in children under the age of five years have found variable median GIA levels, for instance, in a study conducted in Western Kenya, the median GIA levels against three laboratory isolates and one field isolate in children aged less than 12 months were less than 20% (Wilson et al. 2013). In another study, the mean GIA level in children aged 2-4 years was 60% (McCallum et al. 2008)

and the levels were even higher in a separate study which reported 70% GIA in children aged 1-4 years (Dent et al. 2008). Despite the demonstration that young children have antibodies that mediate GIA, it has been inconsistently associated with protection from uncomplicated clinical episodes of malaria or parasitization (reviewed in (Duncan et al. 2012)). Furthermore, no study so far has examined the contribution of GIA to protection against severe episodes of malaria. Antibodies that mediate GIA are acquired at a time when children are at a risk of developing severe malaria and it remains to be determined whether GIA may be protective in this group of children.

For the widely tested merozoite candidates, such as AMA1 and MSP-1, antibodies with potent GIA are efficiently induced following immunization in vaccine trials (Malkin et al. 2005, Mullen et al. 2008) and the assay continues to form the basis for screening the biological activity of antibodies induced by novel blood stage antigens (Douglas et al. 2011, Bustamante et al. 2013, Reddy et al. 2014). However, trials of the most promising blood-stage candidates tested in endemic populations have not proven efficacious (Ogutu et al. 2009, Thera et al. 2011) and protection, if observed at all, was predominantly strain-specific. These findings therefore raise the concern whether GIA serves as an accurate assay for prioritizing vaccine candidates for testing in endemic populations. Furthermore, individuals continuously exposed to infection acquire antibodies that can interfere with the biological activity of neutralizing antibodies (Miura et al. 2008b). There is therefore a need to develop and evaluate other functional assays that better predict protection in malaria exposed individuals, in order to hasten the down-selection of antigens to put forward for clinical testing in endemic populations.

The interaction between antibodies and monocytes and/or neutrophils via Fc receptors expressed on these cells has also been shown to clear circulating parasites either by triggering the release of toxic molecules (Allison and Eugui 1983, Clark and Hunt 1983), soluble

mediators (Bouharoun-Tayoun et al. 1995) or promoting phagocytic uptake and destruction by these cells (Khusmith, Druilhe and Gentilini 1982, Khusmith and Druilhe 1983, Celada, Cruchaud and Perrin 1983, Hill et al. 2012). Assays have been developed that measure the effector function of antibodies in cooperation with monocytes (antibody-dependent cellular inhibition – ADCI assay and opsono-phagocytic assay), or neutrophils (antibody-dependent respiratory burst ADRB assay) in order to evaluate the importance of these mechanisms in predicting *in vivo* protection. Naturally-acquired antibodies against MSP-1 block 2 (Galamo et al. 2009), MSP-3 (Oeuvray et al. 1994), GLURP (Theisen et al. 1998), SERA (Soe et al. 2002) and those induced following immunization with MSP-3 (Druilhe et al. 2005) have been shown to induce ADCI by monocytes. However, this assay has not been widely adopted by many laboratories and its importance as a correlate of protection in longitudinal studies of naturally acquired immunity to malaria, has not been established.

Recently, a reproducible, high throughput opsono-phagocytic assay in the presence of immune sera and merozoites has been developed (Hill et al. 2012) and shown to be strongly associated with protection against uncomplicated clinical episodes of malaria and high-density parasitemia in Papua New Guinean children (Hill et al. 2013). A similar study conducted at the coast of Kenya has also shown that children with antibodies that promote opsono-phagocytosis have a significantly reduced risk of developing uncomplicated malaria (Osier et al. 2014a). These findings however remain to be tested against the clinical endpoint of severe episodes of malaria.

In a study conducted in Gabon, the ability of neutrophils to release high levels of potent reactive oxygen species (ROS) was correlated with enhanced parasite removal in children acutely infected with malaria (Greve et al. 1999). On the other hand, the same group also showed a correlation between severe malaria anemia and the levels of ROS produced by granulocytes (Greve et al. 2000) suggesting that ROS production could also have a detrimental

effect to the host. In their study Greve *et al.*, did not assess the interaction between opsonising antibodies and neutrophils in promoting ROS release as a mechanism of protection, but in a recent study conducted in Senegalese children, an important role of ADRB induced by merozoites opsonized with human serum in protection against uncomplicated clinical episodes of malaria has been demonstrated (Joos et al. 2010).

Although merozoite-specific antibodies are present at very low levels in children aged 0-2 years, the demonstration by several studies that GIA is detectable in some children very early in life provided the rationale for examining whether it may be associated with protection against episodes of severe malaria during the first two years of life. I also adapted the ADRB assay described by Joos *et al.* (Joos et al. 2010) and assessed its role in protection against severe malaria in infants. The importance of these mechanisms in acquisition of immunity against severe malaria in this age group has not yet been demonstrated.

7.1 Objectives

1. To develop and validate the ADRB assay for screening plasma samples obtained from children aged 0-2 years and adults resident in Kilifi County.
2. To examine the acquisition of antibodies that mediate ADRB activity in young children and assess the role of this mechanism in protection against severe malaria during the first two years of life.
3. To compare the growth inhibition activity of plasma samples obtained from children aged 0-2 years and adults resident in Kilifi County using two methodologies.
4. To examine the acquisition of growth inhibitory antibodies in young children and determine their association with protection against severe malaria during the first two years of life.

7.2 Methods

7.2.1 Serum samples

A total of one hundred and ninety five serum samples collected during the three-monthly follow-up visit immediately prior to the disease episode from cases (N=59) and from corresponding matched controls who never developed severe malaria (N=136) were evaluated. A panel of sera from seventy five adults enrolled during cross-sectional surveys conducted in December 2007 (N=25) and December 2009 (N=50) were also tested in parallel to compare responses in adults and infants as described in Section 2.2.3.

7.2.2 GIA and ADRB assays

GIA activity against the 3D7 laboratory adapted parasite line was tested in one or two growth cycles as described in the methods chapter (Section 2.3.2.5.1). Polymorphonuclear cell respiratory burst induced by parasitophorous vacuole enclosed merozoite structures (PEMS), isolated from 3D7 parasites opsonized with plasma from adults and children, was also tested as detailed in the methods chapter (Section 2.3.2.5.3).

7.2.3 Statistical analysis

The Mann-Whitney U test was used to compare levels of GIA and ADRB in the cases versus controls or in infants versus adults. The Kruskal-Wallis test was used to compare continuous variables between more than two groups. Spearman's rank correlation coefficients were used to determine the relationship between two continuous variables. According to the STROBE recommendations (www.strobe-statement.org), information for cases and controls should be given separately when reporting findings of case-control studies and therefore comparisons of

cases and controls are reported separately in this chapter. A conditional logistic regression model was used to compare the odds of developing severe malaria in children who either had GIA or ADRB to that of not developing severe malaria. A P value <0.05 was considered statistically significant.

7.3 Results

7.3.1 ADRB assay development

While the ADRB assay has been well described previously and optimized for large scale testing (Joos et al. 2010), inherent variability between laboratories in i) merozoite isolation, purification, yield and quantification techniques, and ii) donor-dependent activity and yield of neutrophils necessitated independent optimization. Here, I sought to: -

- a) Determine an optimal merozoite isolation technique that gave high ADRB activity and large yields of PEMS suitable for large scale testing.
- b) Quantify the yield of PEMS obtained and standardize the amount of PEMS added in individual wells.
- c) Test the effect of repeated freeze-thawing of PEMS on ADRB activity.
- d) Examine the effect of donor-dependent variability in PMN activity on ADRB activity.
- e) Compare ADRB activity using coated PEMS versus PEMS in solution.
- f) Determine the optimal coating concentration of PEMS.
- g) Determine the optimal serum dilution for use in the assay.
- h) Assess ADRB inter-assay variability.

7.3.1.1 A comparison of ADRB activity induced by merozoites isolated using two techniques.

First, I compared the ADRB levels obtained using merozoites isolated using two published protocols. One protocol yielded parasitophorous vacuole enclosed merozoite structures (PEMS) (Salmon, Oksman and Goldberg 2001) whereas the other yielded pure merozoites (Boyle et al. 2010). Two PEMS preparations that were isolated on different dates were tested in parallel with the pure merozoites. The induction of ADRB activity using neutrophils isolated from a healthy donor was tested against: i) two Kilifi adult serum samples (F031 and F033), ii) a pool of sera from Kilifi adults (PHIS), iii) serum from a donor resident in the UK (UK) and iv) a pool of sera from donors resident in the UK (UK pool). Results are presented as the maximum relative light units (RLU) obtained (absolute RLU values) for every sample tested or indexed according to a positive reference serum, which was included each time the assay was run (indexed RLU). The positive reference serum was a pool of purified IgG obtained from Malawian adults (MIG).

There was a significant reduction in ADRB activity in the presence of non-immune serum (UK and UK pool) compared to immune serum (F031, F033 and PHIS) and this was consistent across the merozoite preparations when assessed using the absolute RLU values (Figure 7.1.1a) and indexed relative to the activity of MIG (Figure 7.1.1b). The ADRB activity obtained using PEMS (blue bars) and pure merozoites (green bars) isolated on the same day were higher than that of PEMS that had been prepared on a separate day (red bars). In this experiment, the number of PEMS or merozoites added to each well was not quantified and this could also explain the differences in ADRB activity.

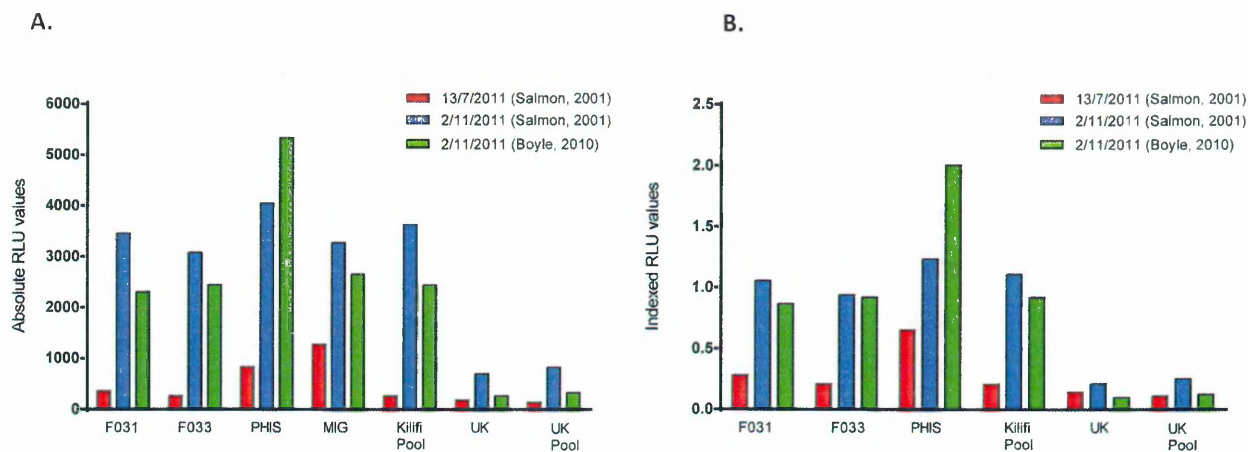


Figure 7.1.1 ADRB activity induced by three different merozoite preparations against a panel of sera. Red, blue and green bars represent PEMS (isolated and frozen on 13/7/2011), PEMS (isolated and frozen on 02/11/2011) and pure merozoites (isolated and frozen on 02/11/2011). Merozoite opsonised with i) sera from malaria exposed individuals resident in Kilifi (F031 and F033), ii) pooled serum from a panel of malaria-exposed individuals (PHIS), iii) sera from a non-exposed individual resident in the UK (UK) and iv) pooled serum from a panel of non-exposed individuals (UK pool) were examined. Absolute RLU values are shown in (A) and values indexed relative to an IgG pool from Malawian adults (MIG) as shown in (B). Individual bars represent the mean of duplicates of one assay.

7.3.1.2 Quantifying the number of PEMS isolated and added to individual wells.

To standardize the number of PEMS added to each well, two PEMS were prepared on separate days (2/11/2011 and 16/11/2011) and the number of schizonts enumerated prior to freezing as described in the methods chapter (Section 2.3.2.4). Approximately 1.85×10^5 schizonts/ml of each PEMS preparation was added to each well and tested against a panel of serum. In parallel pure merozoites isolated as previously described (Boyle et al. 2010) were also tested but I could not conclusively enumerate the number of pure merozoites isolated as outlined by Boyle *et al.*, as the merozoite pellet obtained was very small.

In two separate experiments, there was significant variability in ADRB activity between the two PEMS batches (Figure 7.1.2a and Figure 7.1.2b). In Figure 7.1.2a, ADRB activity induced by pure merozoites was consistently lower than that of the two PEMS preparations but in Figure 7.1.2b, there was no consistent pattern in ADRB activity between the merozoite preparations. The ADRB activity for individual samples in the second experiment was relatively lower compared to the first experiment. For example, there was a three-fold reduction in ADRB activity for sample F033 in the second experiment (0.62 versus 0.23 RLU). In addition, reactivity of the UK serum pool (HSP) was similar to the malaria exposed serum samples in both experiments. It was not clear why I was not seeing differences in ADRB activity between the malaria exposed and non-exposed samples and the following set of experiments were conducted to evaluate the conditions that could explain these findings, with the aim of enhancing ADRB activity of malaria exposed individuals.

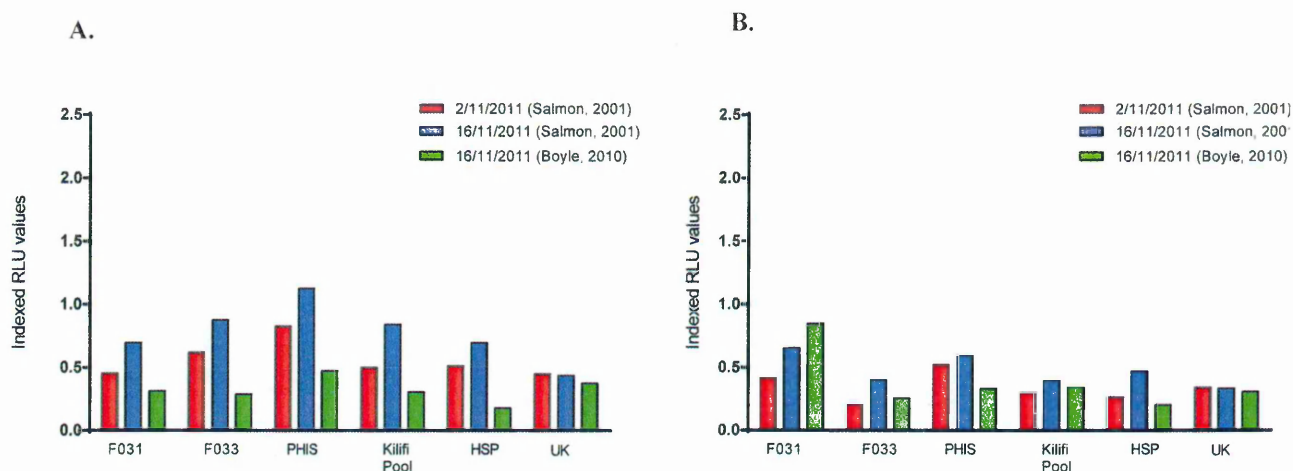


Figure 7.1.2 ADRB activity induced by PEMS frozen at a concentration of 1.85×10^5 schizonts/ml and pure isolated merozoites in two independent experiments. Sera from i) malaria exposed individuals resident in Kilifi (F031 and F033), ii) pooled serum from a panel of malaria-exposed individuals (PHIS), iii) sera from a non-exposed individual resident in the UK (UK) and iv) pooled serum from a panel of non-exposed individuals (HSP) were examined. The experiments were conducted on two separate days (A) 29/11/11 and (B) 13/12/2011

7.3.1.3 The effect of repeated freeze-thawing on ADRB activity

A possible explanation for reduced ADRB activity by serum from malaria-exposed donors could be the degradation of PEMS as a result of repeated freeze-thawing. To test the effect of freeze-thawing on ADRB activity, PEMS were isolated and tested following one, two or three freeze-thaw cycles in the presence of malaria-exposed (PHIS and Kilifi pool) and non-exposed sera (UK and HSP). An aliquot of the PEMS was also frozen in glycerol and tested following one freeze-thaw cycle. For the Kilifi pool and MIG samples, there was a decrease in absolute RLU values with increase in the number of freeze thaw cycles (Figure 7.1.3a). However, when the absolute values were indexed relative to MIG, there was no significant difference in ADRB activity induced by PEMS that had undergone one, two or three freeze-thaw cycles (Figure 7.1.3b) suggesting that the PEMS were still intact after several freeze thaw cycles. PEMS frozen in glycerol induced a lower respiratory burst. Variability in ADRB activity between the two PEMS preparations was also noted in this experiment despite adding a similar concentration of PEMS in each well.

Although ADRB activity induced by malaria non-exposed sera was lower compared to malaria exposed sera, I could not reproduce the high ADRB activity induced by malaria exposed serum as was previously observed in Figure 7.1.1 (indexed RLU values were 0.5, 0.6, 0.5 for F031, PHIS and a Kilifi serum pool compared to 1.05, 1.23 1.11, respectively) (Figure 7.1.1 and Figure 7.1.3).

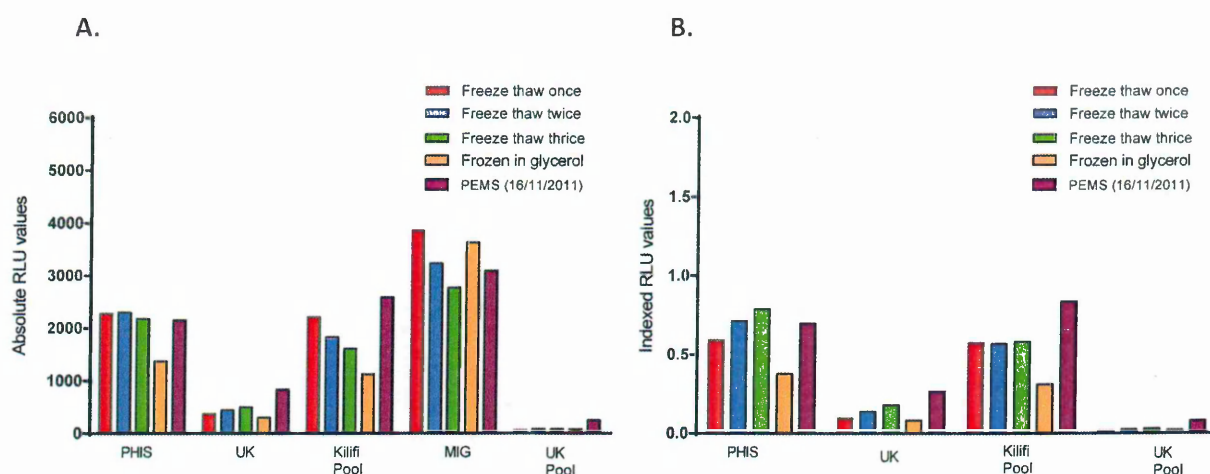


Figure 7.1.3. The effect of repeated freeze-thawing of PEMS on ADRB activity. PEMS were isolated and tested in the assay following one (red bars), two (blue bars) and three (green bars) freeze-thaw cycles. In comparison, PEMS were frozen in glycerol (orange bars) and tested after one freeze-thaw cycle. A batch of PEMS tested in a previous experiment (Purple bars) was also included to compare ADRB activity from different batches. Two serum pools from malaria-exposed individuals (PHIS and Kilifi pool), sera from a non-exposed individual resident in the UK (UK) and pooled serum from a panel of non-exposed individuals (HSP)

7.3.1.4 The effect of donor dependent variability on ADRB activity

Next, the variability in ADRB activity by polymorphonuclear cells (PMNs) obtained from different donors was examined. PMNs were isolated from three healthy donors and tested in the presence of a pool of IgG from Malawian adults (MIG) and a pool of sera from UK adults (UK). Two PEMS preparations used in the previous experiment were also tested to compare batch to batch variability.

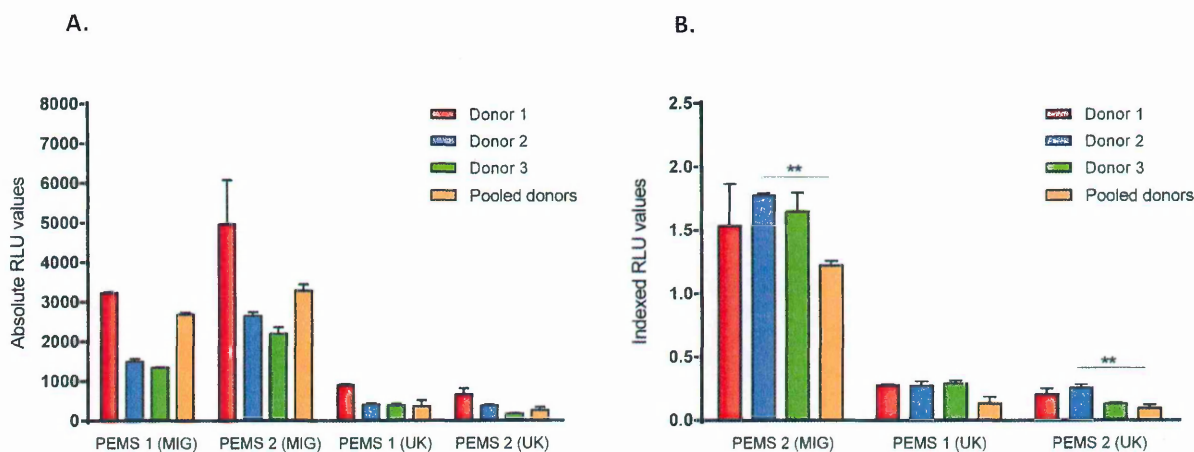


Figure 7.1.4. ADRB induction by PMNs obtained from different donors. PMNs isolated from three individual donors and a pool prepared by mixing the three donor PMNs were tested in the assay against two PEMS preparations (PEMS1 and PEMS2) opsonized with IgG from a pool of Malawian adults (MIG) and serum from a non-exposed individual (UK). The absolute values (maximum relative light units) are shown in figure (A) and values indexed relative to reactivity of MIG on PEMS 1 are shown in figure (B).

Based on the absolute values, there was substantial donor dependent variability in absolute RLU values obtained using PMNs from different donors either against malaria exposed or non-exposed serum (Figure 7.1.4a). PMNs from donor 1 had the highest absolute RLU values (blue bars) whereas those obtained from donor 3 had the lowest values (green bars) across the samples tested. Absolute RLU values obtained using PMNs pooled from the three donors had ADRB activity second to PMNs from donor 1 (Figure 7.1.4a). When the ADRB activity of different PMN donors was examined relative to the activity of MIG on PEMS 2, there was no significant difference between the three donors' PMNs for the two serum samples tested (Figure 7.1.4b). However, the indexed ADRB activity of pooled PMNs was lower than the individual donors' PMNs and the difference was significant when compared against donor 2's PMNs. Based on these findings, different PMN donors could be used on different days without eliciting significant variability in the assay.

7.3.1.5 Comparing ADRB activity of coated PEMS versus PEMS in solution

In order to enhance the ADRB activity induced by malaria-exposed serum, I next compared the reactivity of PEMS in solution versus coated PEMS. The rationale for testing this was based on previous optimization steps demonstrating that antigen in solution does not induce respiratory burst in the presence of immune human serum (David Llewellyn, PhD thesis) and mouse sera (Llewellyn et al. 2014).

ADRB activity induced by PEMS in solution, coated PEMS, coated recombinant MSP-1₁₉ protein and ovalbumin (a non-malaria protein) opsonized with malaria non-exposed (HSP, UK1 and UK5) or malaria exposed sera (PHIS and F042) were tested. The level of ADRB by malaria-specific antigens was significantly lower in malaria non-exposed versus exposed adults (Figure 7.1.5). ADRB activity induced by coated PEMS was higher than the reactivity

of PEMS in solution suggesting that there is enhanced co-localization of Fc receptors on the surface of PMNs when PEMS are coated onto the plate as opposed to floating in solution.

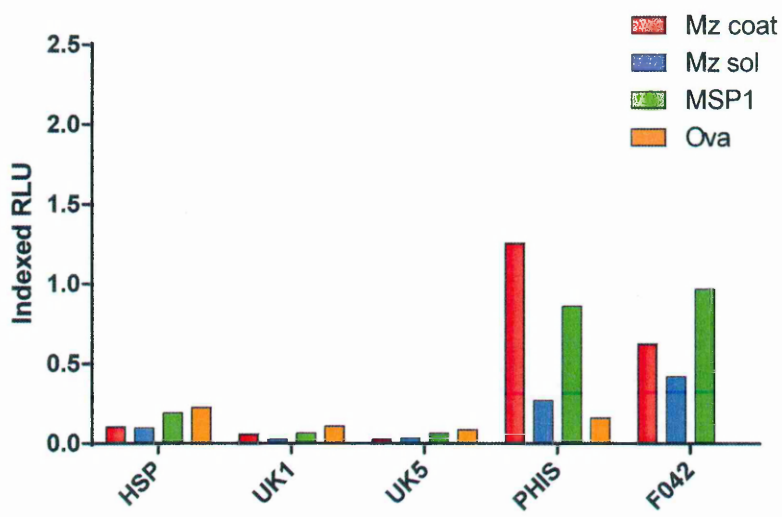


Figure 7.1.5 A comparison between ADRB activity induced by coated PEMS versus PEMS in solution. ADRB activity induced by malaria exposed (PHIS and F042) and non-exposed (HSP, UK1 and UK5) sera against PEMS coated onto a plate (red bars), PEMS in solution (blue bars), coated recombinant MSP-1₁₉ protein (green bars) and ovalbumin (orange bars). Values are indexed relative to MIG.

7.3.1.6 Determining the optimal coating concentration of PEMS preparations

Having established that PEMS coated onto a plate induce higher ADRB activity compared to PEMS in solution, the optimal coating dilution of PEMS in the assay was determined by titrating PEMS at two doubling dilutions starting at 1.85×10^5 schizonts/ml. In the malaria exposed serum samples (F008, F042 and F070), the highest ADRB activity was induced by PEMS coated at 1.85×10^5 schizonts/ml (neat PEMS). There was no significant difference in ADRB activity by PEMS diluted 10-fold or 20-fold for all samples tested with the exception of serum sample F042 (Figure 7.1.6).

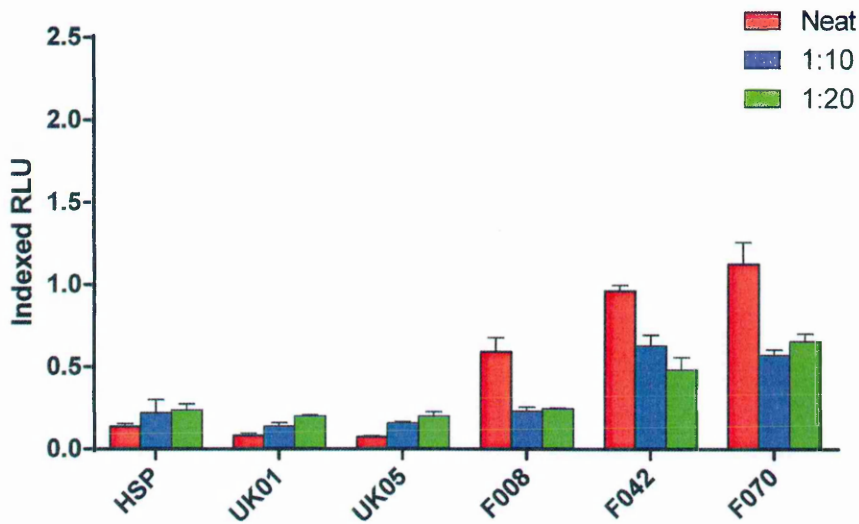


Figure 7.1.6 Determining the optimal coating concentration of PEMS. ADRB activity induced by malaria exposed (F008, F042 and F070) and non-exposed (HSP, UK01 and UK05) sera against doubling dilutions of PEMS coated onto a plate. Two-fold doubling dilutions were tested starting at 1.85×10^5 schizonts/ml.

7.3.1.7 Determining the optimal serum dilution

The optimal serum dilution was also determined by titrating a pool of serum from malaria-exposed adults (Kilifi pool) starting at 1:25 to 1:1000 dilution in PBS (Figure 7.1.7). The dynamic range of serum dilution was between 1:25 to 1:100, therefore a mid-range dilution of 1:50 was ideal in this assay to accurately distinguish between responders versus non-responders.

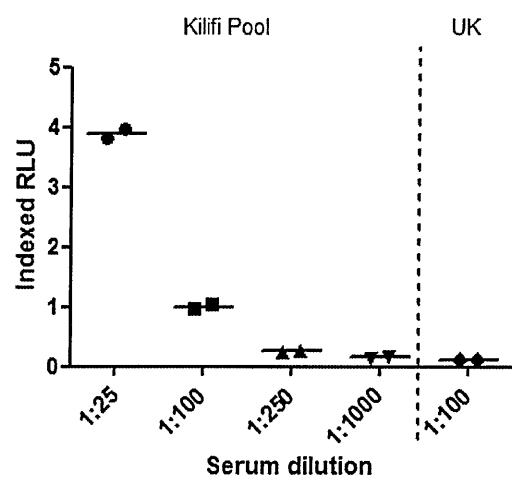


Figure 7.1.7. Determining the optimal serum dilution. A pool of serum from malaria exposed adults (Kilifi pool) was tested at increasing serum dilutions (1:25 to 1:1000). In parallel, the ADRB activity of a serum sample from a non-exposed donor was tested at 1:100 dilution. Values of duplicate wells are shown.

7.3.1.8 Determining ADRB inter-assay variability

To assess the level of variability when the assays were conducted on different days, a panel of sera was tested for induction of ADRB activity in five separate experiments using neutrophils from different donors. There was significant inter-assay variability for most samples tested (Figure 7.1.8). Despite this observation, the inherent differences between samples were consistent between experiments and with the exception of sample K6, the majority of the malaria exposed individuals were consistently classified as being ADRB positive (based on a cutoff of mean plus three standard deviations of the seven UK sera tested across five experiments). Only one malaria exposed serum sample was ADRB negative (K4) and this was consistent across all the experiments. 6/7 (85%) of all the malaria non-exposed sera were classified as ADRB negative across the five experiments.

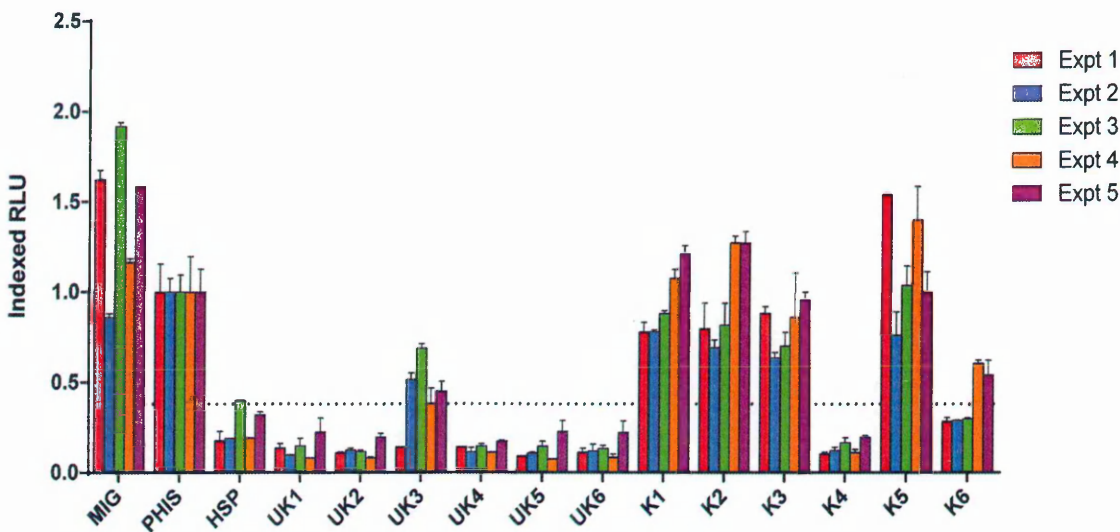


Figure 7.1.8 ADRB assay reproducibility. Serum samples obtained from six malaria exposed adults (K1 to K6), serum and IgG pooled from exposed adults (PHIS and MIG, respectively), six serum samples from malaria non-exposed individuals (UK1 to UK6) and a pool of serum from non-exposed adults (HSP) were assayed on five separate days to assess inter-assay variability. The values are indexed relative to a pool of IgG from Kilifi adults (PHIS) and individual bars indicate the mean activity of two assay replicates.

The optimal set of parameters established for subsequent experiments were as follows: -

- (i) Preparation of a single PEMS batch sufficient to test all samples in a cohort since I could not resolve the variability observed when different PEMS batches were tested.
- (ii) Coating PEMS onto the plate at 1.85×10^5 schizonts/ml concentration followed by an overnight incubation at room temperature.
- (iii) Addition of individual serum samples diluted at 1:50 in PBS and assayed in duplicate.
- (iv) Addition of 50 μ l of 5×10^5 PMNs isolated from different donors.

7.3.2 Comparison of ADRB activity between malaria-exposed children and adults versus malaria non-exposed adults

To determine the range of responses observed in young children and adults resident in Kilifi county, the level of ADRB induced by serum from malaria-exposed adults (N=100), infants aged 0-2 years recruited into a birth cohort (N=195) and malaria naïve adults enrolled in Phase Ia malaria vaccine trials at the Jenner Institute (N=13) were compared. There was a significant difference in ADRB activity between malaria-exposed adults and children ($P < 0.001$) and between malaria-exposed and non-exposed adults ($P < 0.001$) (Figure 7.2). However no significant difference in ADRB activity was noted between malaria-exposed children and malaria non-exposed adults ($P = 0.376$).

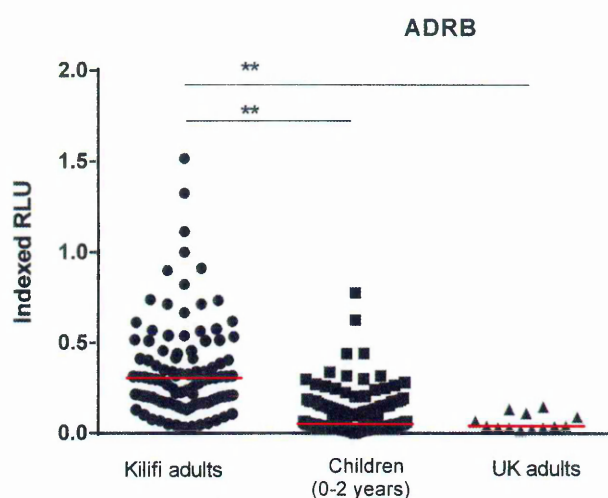


Figure 7.2 Comparison of ADRB levels induced by serum obtained from adults resident in Kilifi county (dark circles), children aged 0-2 years (dark squares) and malaria naïve adults (dark triangles). Horizontal red lines indicate the median ADRB level for each group. Values are indexed relative to MIG.

7.3.3 Age-specific ADRB activity in infants aged 0-2 years

Overall, the highest median ADRB activity was observed in plasma samples collected from infants aged 0-3 months. Thereafter, there was a significant decline in ADRB activity upto 6 months of age followed by a modest but gradual increase with age upto 28 months (Kruskal Wallis test; $P < 0.0001$) (Figure 7.3a). A similar trend was observed when ADRB activity by age was grouped into children who subsequently developed severe malaria during the first two years of life (Kruskal Wallis test; $P = 0.334$) and those who did not (Kruskal Wallis test; $P < 0.0001$) (Figure 7.3b).

Age-specific comparisons of ADRB activity in cases versus controls revealed no significant differences between the two groups although the median ADRB levels were consistently higher in the cases compared to controls (Figure 7.3b)

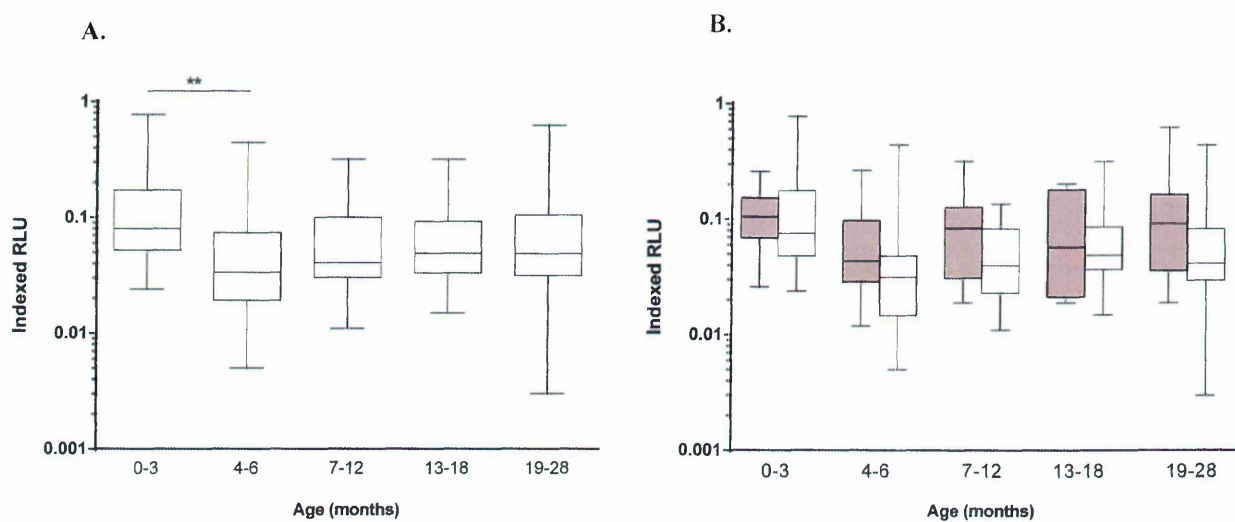


Figure 7.3. Age-specific ADRB levels in infants aged 0-2 years. The box plots represent (A) the overall range of responses observed in children grouped into five age categories (0-3, 4-6, 7-12, 13-18 and 19-28 months) and (B) responses in children who developed severe malaria during the first two years of life (grey bars) and their corresponding controls who did not develop severe malaria (white bars). ** P-value <0.05.

7.3.4 Correlation between ADRB activity and antibody titres against specific antigens in adults

The correlations between ADRB levels and ELISA OD values against AMA1 (HB3), MSP-2(Dd2), MSP-3(3D7) and the R0 fragment of GLURP were assessed. For all antigens tested, there was a significant positive correlation between ADRB activity and antibody levels (correlation coefficients 0.31, 0.39, 0.59 and 0.40 for AMA1 (HB3), MSP-2(Dd2), MSP-3(3D7) and GLURP-R0, respectively) (Figure 7.4).

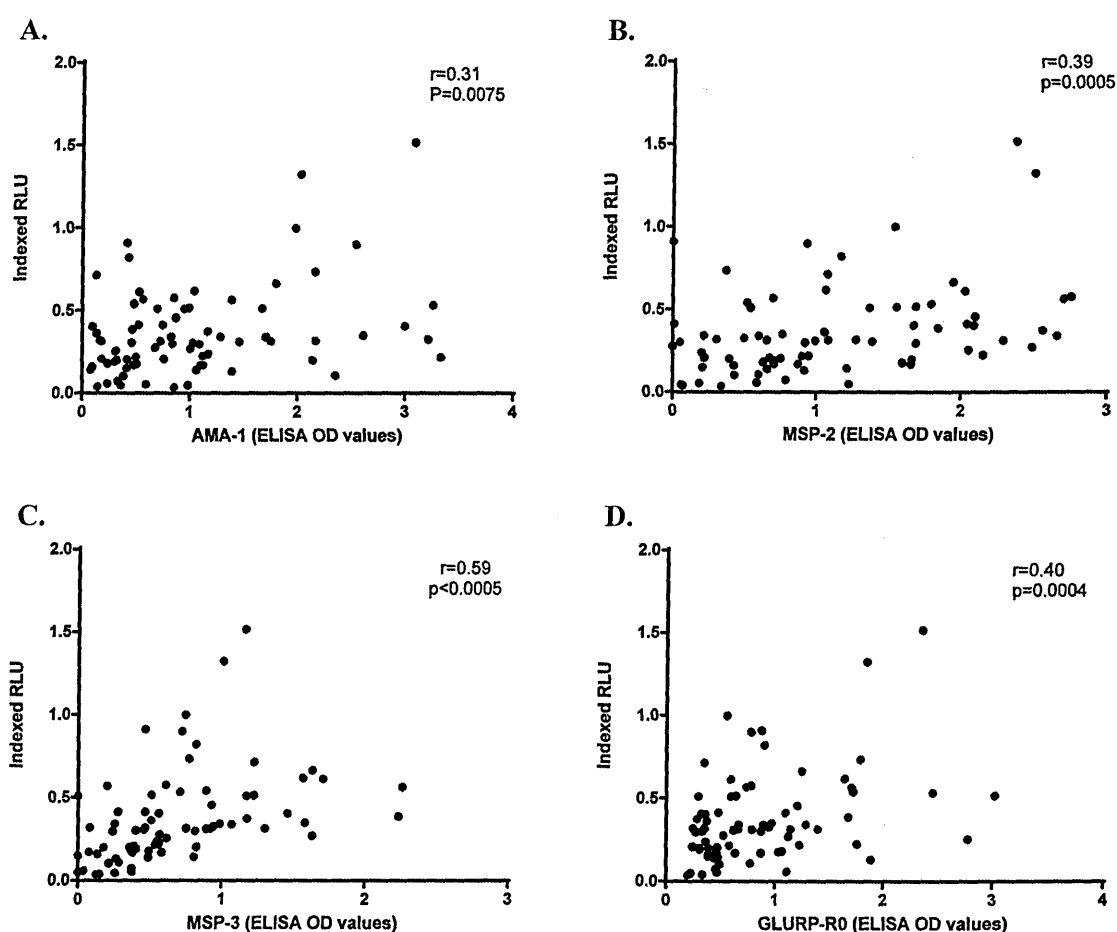
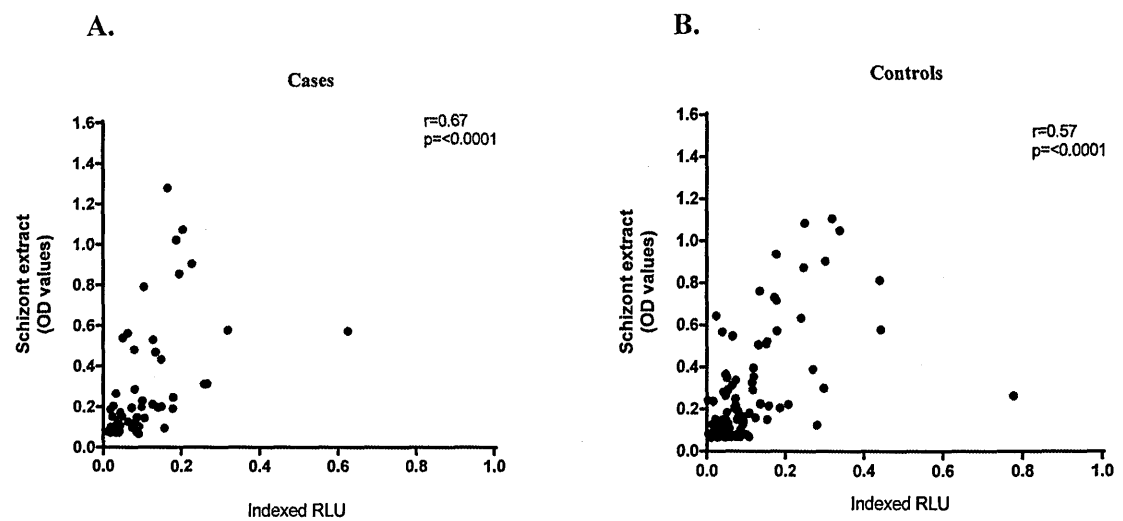
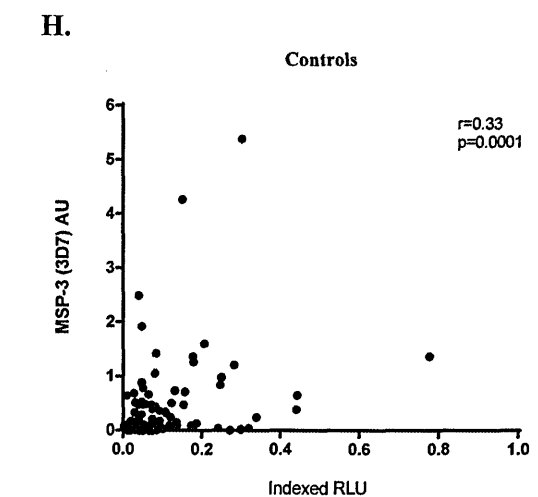
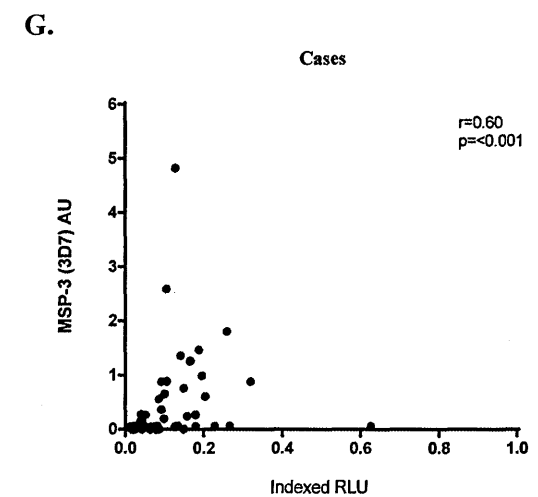
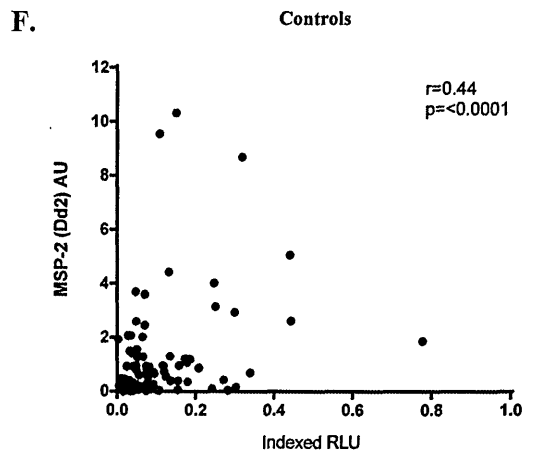
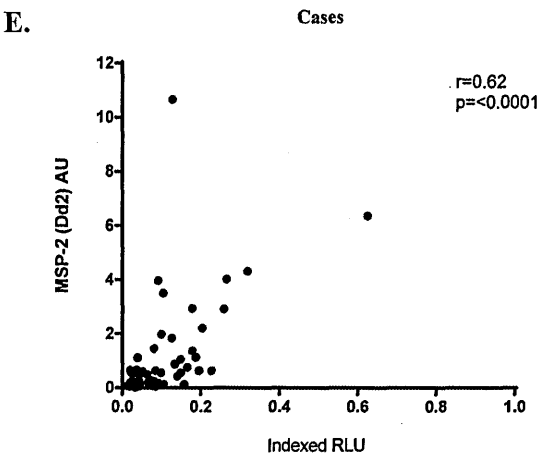
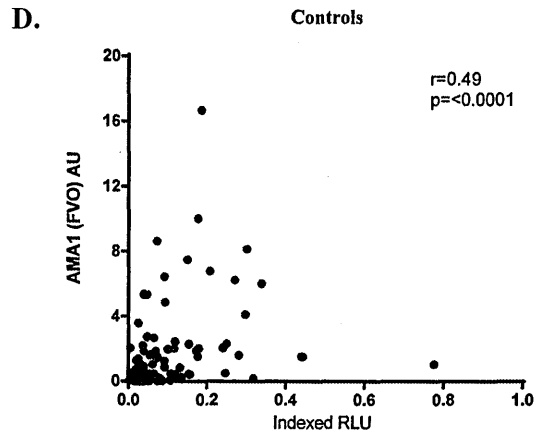
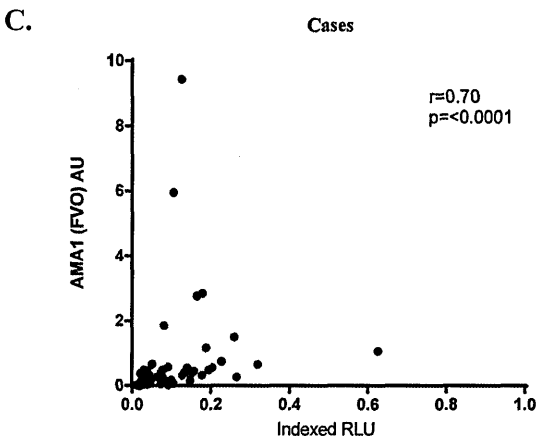


Figure 7.4. Correlation analysis between ADRB levels and antibody titres against (A) AMA1 (HB3), (B) MSP-2 (Dd2), (C) MSP-3 (3D7) and (D) GLURP-R0 fragment measured in plasma samples collect from adults resident in Kilifi (N=75). Correlation coefficients were determined by Spearman's rank test. $P<0.05$ was considered statistically significant.

7.3.5 Correlation between ADRB activity and antibody titres to specific antigens in children who developed severe malaria (cases) and those who did not (controls).

The correlations between levels of ADRB and IgG to specific antigens were determined separately for the cases and controls. Plasma samples collected at the three-monthly time point that was closest to the disease episode for the severe malaria cases (N=59) and corresponding controls (N=147) were analysed. For all antigens tested, there was a positive correlation between ADRB activity and antibody titres in the cases (Correlation coefficients 0.67, 0.70, 0.62, 0.60, 0.18, 0.21 for schizont extract, AMA1, MSP-2, MSP-3, MSP-1₁₉ and *Pf*Rh2, respectively) and controls (correlation coefficients were 0.57, 0.49, 0.44, 0.33, 0.20, 0.04 for schizont extract, AMA1, MSP-2, MSP-3, MSP-1₁₉ and *Pf*Rh2, respectively). The correlation between ADRB and MSP-1₁₉ and *Pf*Rh2 antibodies was weak and not statistically significant in both the cases and controls (correlation coefficients were 0.18, 0.21 in the cases and 0.20 and 0.04 in the controls for MSP-1₁₉ and *Pf*Rh2 antibodies, respectively) (Figure 7.5).





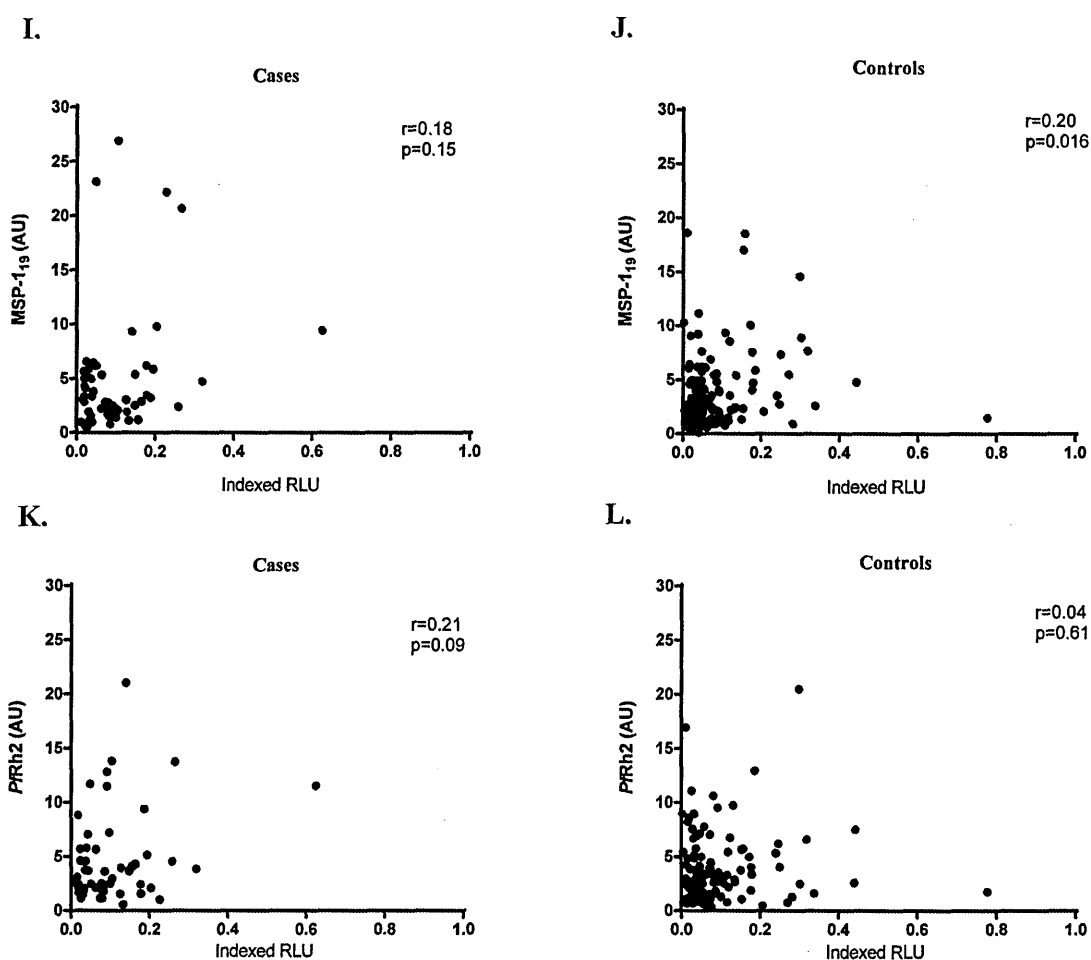


Figure 7.5 Correlation analysis between antibody titres to specific antigens and ADRB activity in children who developed severe malaria (cases) versus their corresponding controls. Scatter plots show the relationships between titres against schizont extract, AMA1, MSP-2, MSP-3, MSP-1₁₉, *Pf*Rh2 and ADRB activity of the plasma samples of cases (A, C, E, G, I and K) and controls (B, D, F, H, J and L), respectively.

7.3.6 Comparing growth inhibition of *P. falciparum* 3D7 parasites by human sera in a one-growth cycle assay (pLDH detection) versus a two-growth cycle assay (flow cytometry)

The ability of serum obtained from adults resident in Kilifi county (N=75) and children aged 0-2 years (N=189) to inhibit growth of 3D7 parasites *in vitro* was assessed using two methodologies; i) flow cytometry analysis of trophozoite stage parasites stained with ethidium bromide after two growth cycles or ii) quantification of parasite lactate dehydrogenase (pLDH) enzyme production after a single growth cycle. GIA levels were significantly higher in the 2-growth cycle assay compared to the single cycle assay in serum samples collected from the children (median GIA levels were 7.75% and 10.56% by pLDH and flow cytometry, respectively) (Figure 7.6.1a). A three-fold increase in median growth inhibition level was also noted in the adult samples (median GIA levels of 6.19% versus 19.98% by pLDH and flow cytometry, respectively) (Figure 7.6.1b).

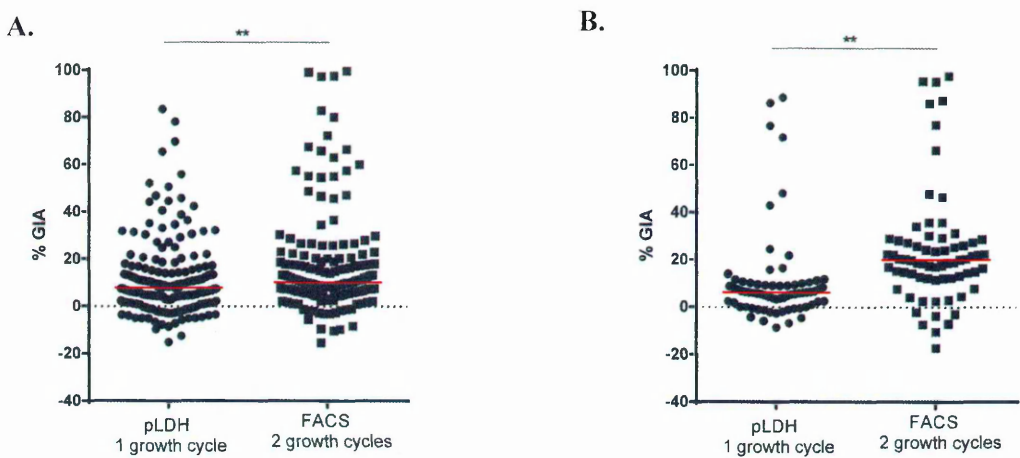


Figure 7.6.1 A comparison between GIA levels assessed by measuring pLDH production following a single growth cycle (black circles) or flow cytometry analysis of ethidium bromide stained parasites following two growth cycles (black squares) in (A) a subset of KBC children aged 0-2 years (N=189) and (B) adults resident in Kilifi county (N=75). Horizontal red lines represent the median GIA levels of each group.

The correlation between GIA levels determined either by measuring pLDH production or by flow cytometry was determined using the spearman's rank test. There was a strong positive correlation between GIA activity by both methods in plasma samples collected from children aged 0-2 years (correlation coefficient, 0.65, $P<0.0001$) (Figure 7.6.2a) and adults resident in Kilifi (Correlation coefficient, 0.72, $P<0.0001$) (Figure 7.6.2b)

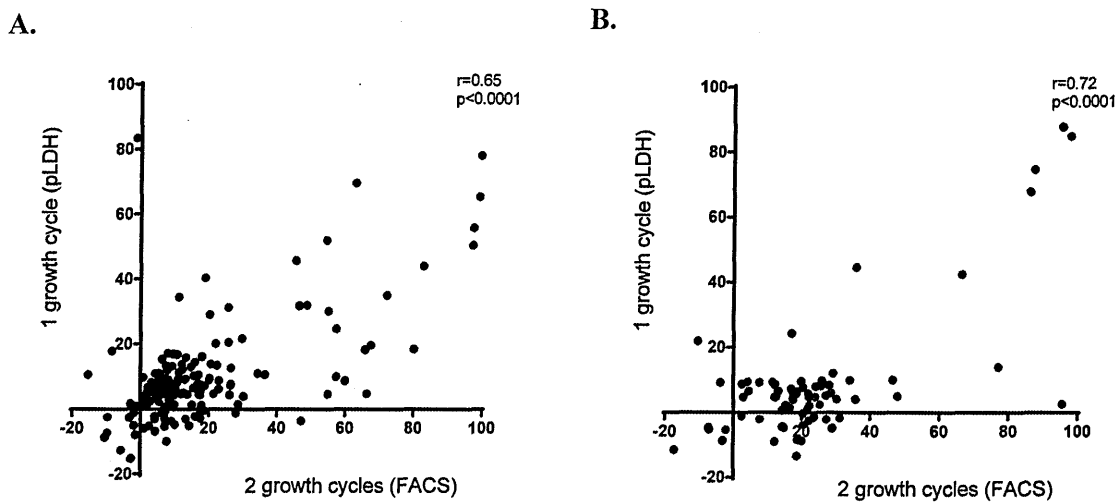


Figure 7.6.2. Correlation analysis between growth inhibition determined by measuring pLDH production after one growth cycle (y-axis) or by flow cytometry after two growth cycles (x-axis) in plasma samples collected from children aged 0-2 years (A) and adults resident in Kilifi county (B). Scatter plots show the correlation between results obtained using the two methodologies. The spearman's rank test was used to calculate the correlation coefficients.

A comparison of GIA levels by plasma obtained from children, malaria exposed and naïve adults revealed significantly higher GIA levels in plasma from children compared to malaria exposed and naïve (UK) adults using pLDH detection after a single growth cycle (Figure 7.6.3a). In contrast, plasma from malaria-exposed adults elicited a significantly higher median GIA level compared to plasma from children and UK adults after two growth cycles (Figure 7.6.3b)

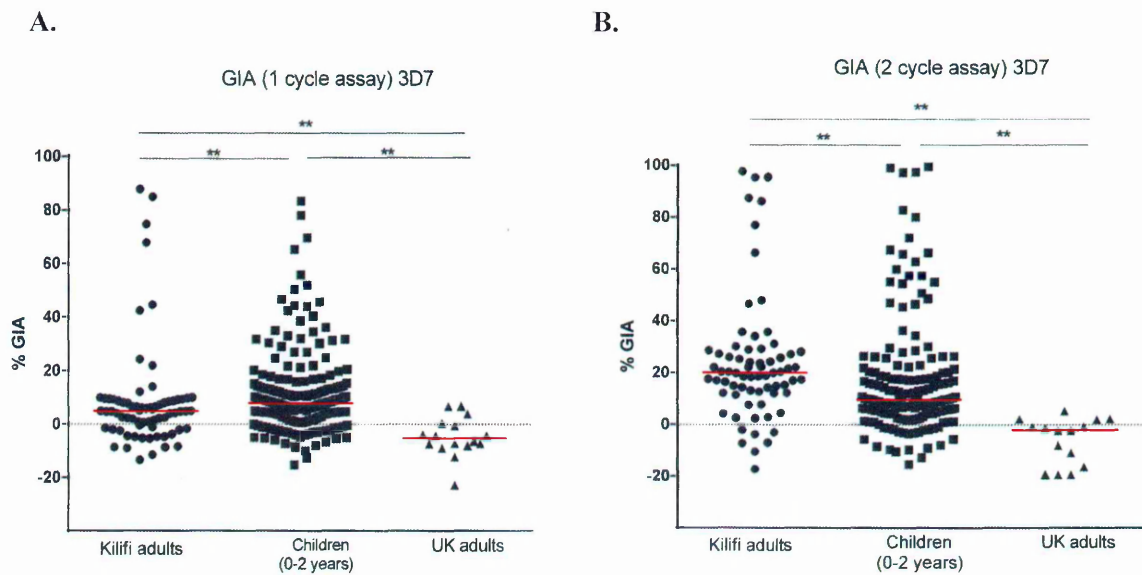


Figure 7.6.3 Distribution of GIA levels of plasma obtained from adults resident in Kilifi County (black circles), children aged 0-2 years recruited into a birth cohort (black squares) and malaria naïve adults resident in the UK (black triangles). Comparisons between GIA assessed by pLDH measurement after a single growth cycle (A) and flow cytometry analysis of mature parasites after two growth cycles (B) are shown. ** represents P values <0.05.

7.3.7 Age-specific growth inhibition levels in children aged 0-2 years.

Comparisons of GIA levels among children aged 0-2 years revealed no significant difference in median GIA levels with increase in age (Kruskal Wallis test; $P=0.112$) (Figure 7.7a). The levels were highest in children aged 0-3 months. A similar trend was also observed when GIA levels in the different age groups were categorized into children who developed severe malaria ($P=0.86$) and those who did not (Kruskal Wallis; $P=0.07$) (Figure 7.7b). The median GIA levels between the cases and controls were similar across all age-categories (Figure 7.7b).

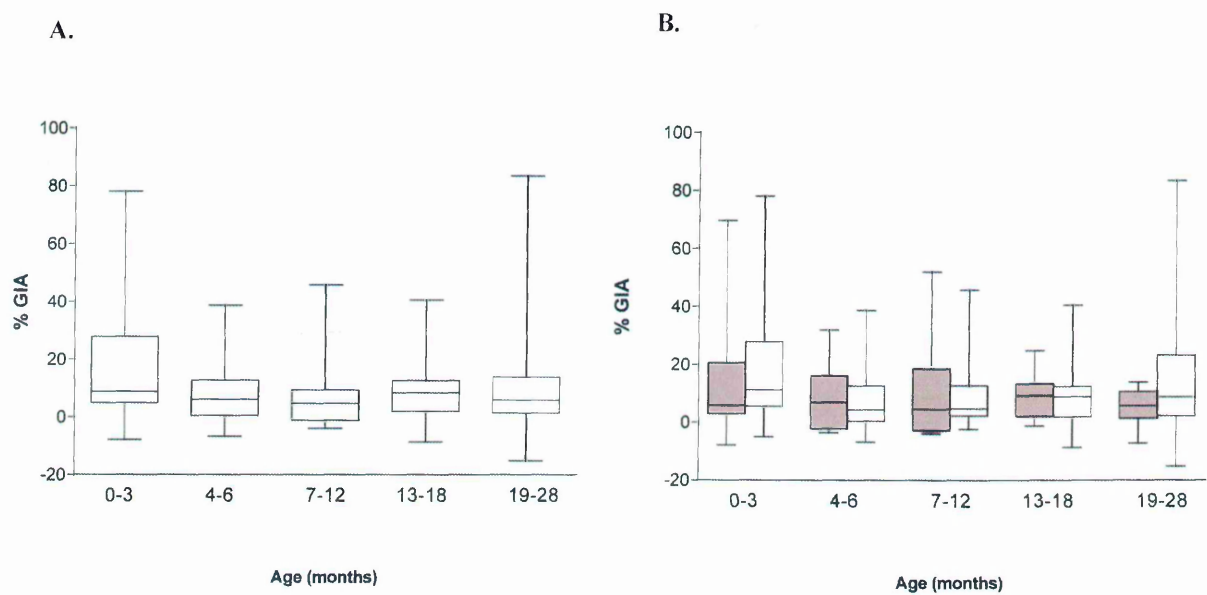


Figure 7.7. Age-specific GIA levels in infants aged 0-2 years. The box plots represent (A) the overall range of responses observed in children grouped into five age categories (0-3, 4-6, 7-12, 13-18 and 19-28 months) and (B) responses in children who developed severe malaria during the first two years of life (grey bars) and their corresponding controls who did not develop severe malaria (white bars). ** P -value <0.05 . Data obtained from the two-cycle GIA.

7.3.8. Association between GIA and antibody titres against specific antigens in adult serum samples

GIA levels obtained from the two growth cycle assay were used to assess the relationship between GIA and IgG levels against AMA1 (HB3), MSP-2(Dd2), MSP-3(3D7) and the R0 fragment of GLURP protein (GLURP-R0) in adult serum samples. There was a weak and non-significant association between growth inhibition levels and antibody titres to all the antigens tested (correlation coefficients obtained were 0.11, -0.01, 0.01 and 0.02 for AMA1 (HB3), MSP-2(Dd2), MSP-3(3D7) and GLURP-R0, respectively, P values >0.05) (Figure 7.8). Similar associations were observed using GIA data assessed by quantifying pLDH production in a one growth cycle assay (Correlation coefficients 0.03, -0.02, 0.11 and -0.01 for AMA1 (HB3), MSP-2(Dd2), MSP-3(3D7) and GLURP-R0, respectively, P values >0.05).

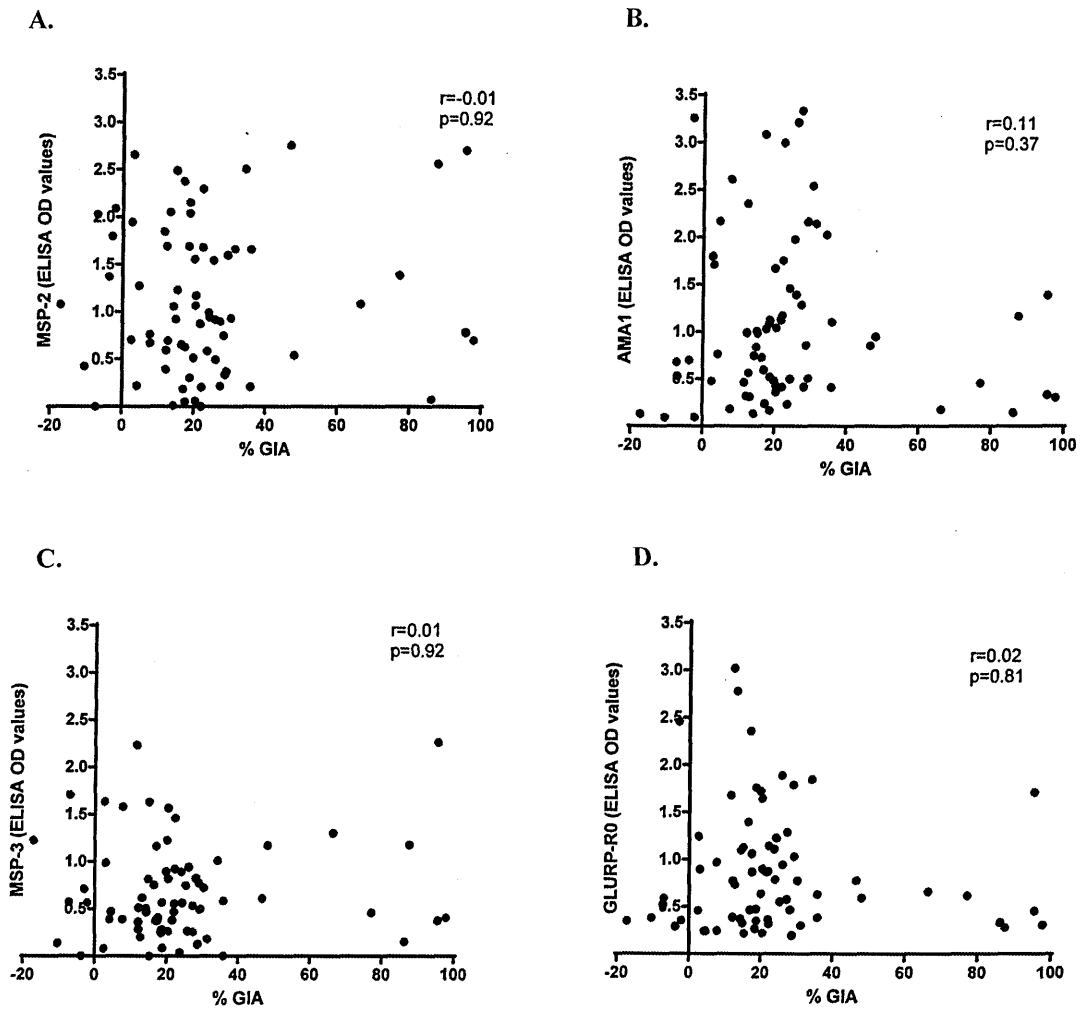
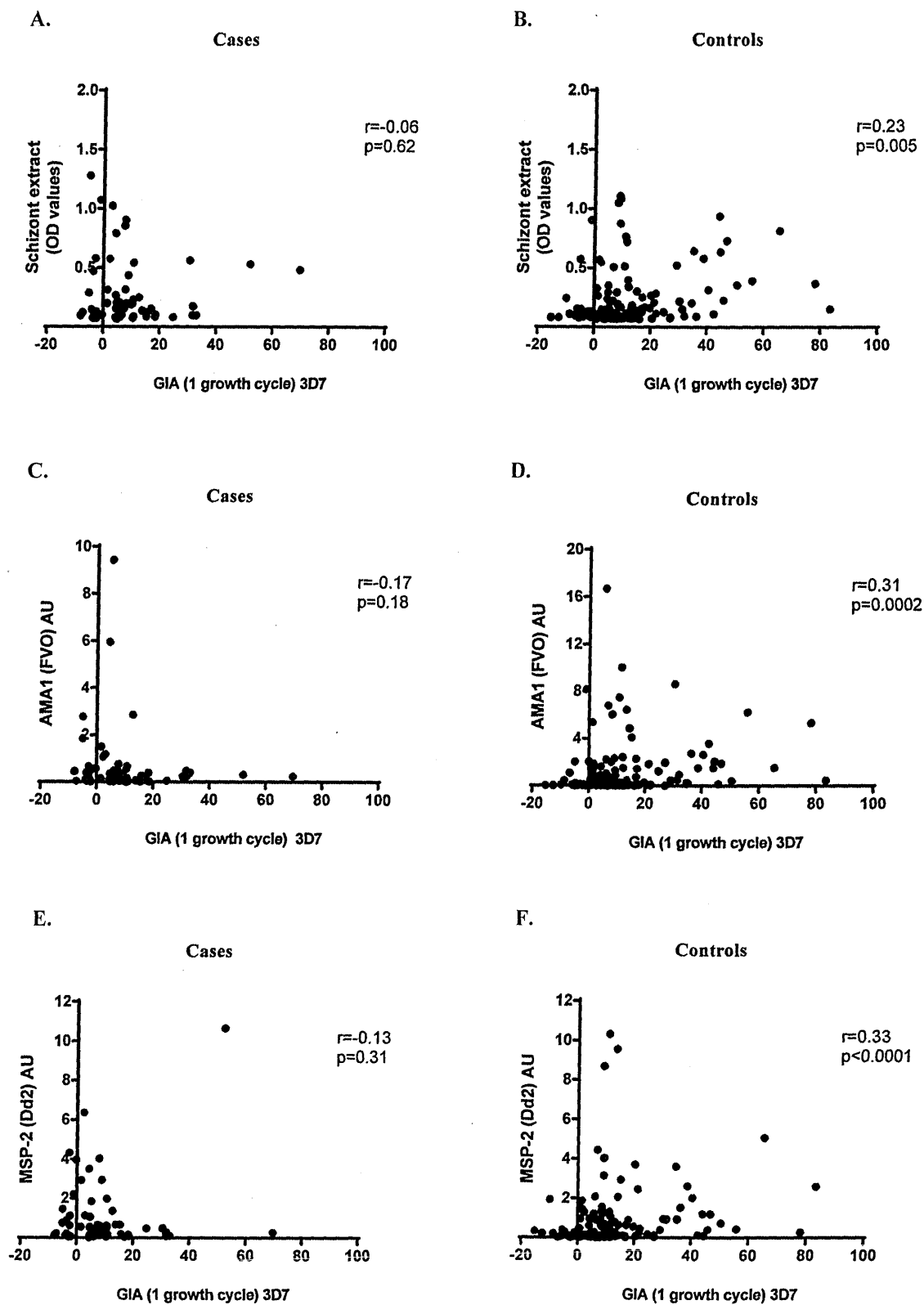


Figure 7.8 Correlation analysis between GIA levels quantified by flow cytometry in a two growth cycle assay and antibody titres against (A) AMA1 (HB3), (B) MSP-2 (Dd2), (C) MSP-3 (3D7) and (D) GLURP-R0 fragment measured in plasma samples collected from adults resident in Kilifi (N=75). Correlation coefficients were determined by Spearman’s rank test. $P < 0.05$ was considered statistically significant.

7.3.9 Association between GIA and antibody titres against specific antigens in plasma samples collected from children aged 0-2 years.

Serum samples collected from children aged 0-2 years were grouped into those who subsequently developed severe malaria (N=59) and those who did not (N=147). Similar to the findings in adults (Figure 7.9), the correlation between GIA levels and antibody titres against AMA1, MSP-2, MSP-3, MSP-1₁₉ and *Pf*Rh2 was weak and only evident among the controls (Figure 7.9).



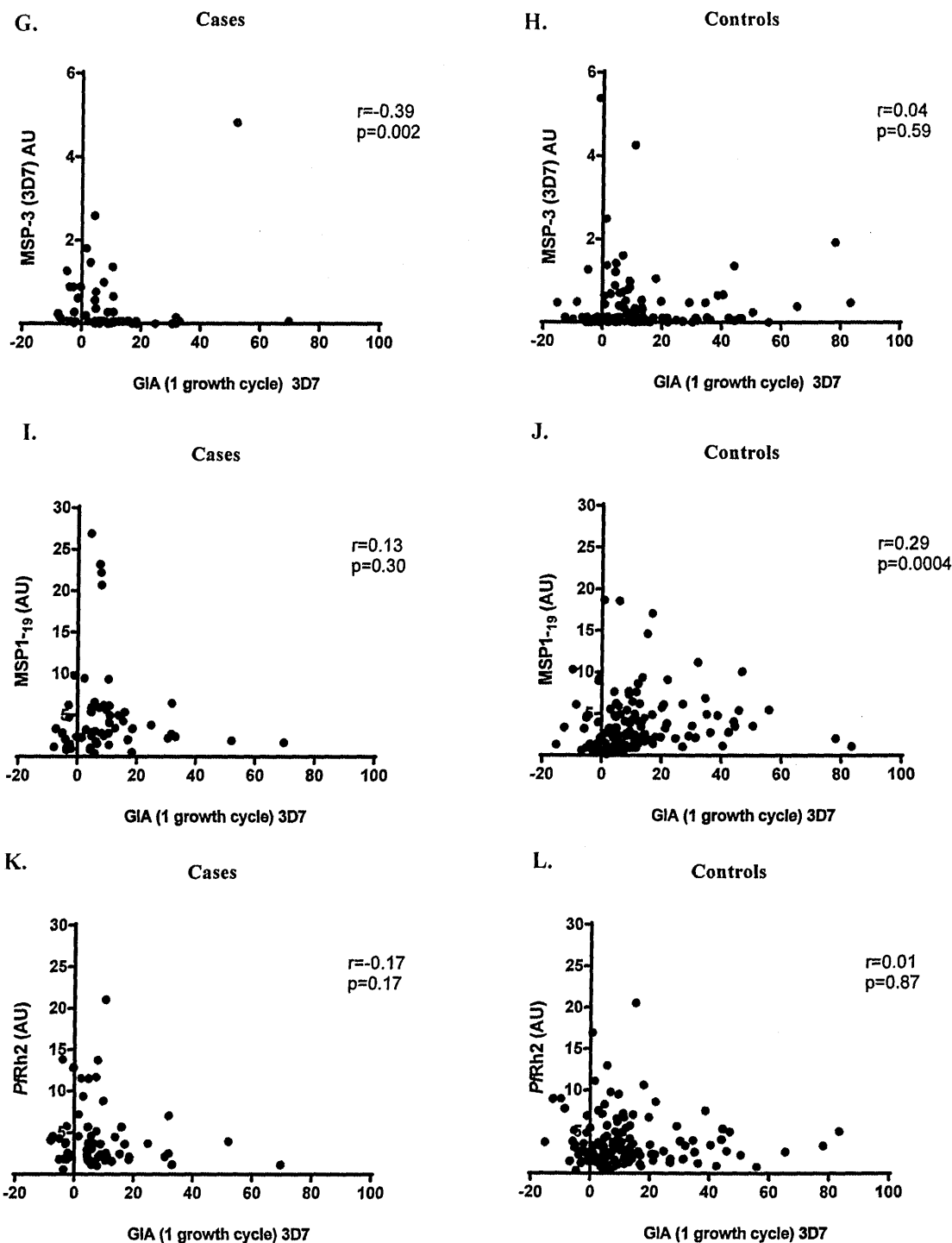


Figure 7.9 Correlation analysis between antibody titres to specific antigens and GIA activity measured in a one growth cycle assay in children who developed severe malaria (cases) versus their corresponding controls. Scatter plots show the relationships between titres against schizont extract, AMA1, MSP-2, MSP-3, MSP-1₁₉, *Pf*Rh2 and GIA levels of the plasma samples of cases (A, C, E, G, I and K) and controls (B, D, F, H, J and L), respectively.

7.3.10 Correlation between GIA and ADRB

The correlation between the 2-growth cycle assay GIA and ADRB was examined in both children and adults. There was no correlation between both assays when examined in serum samples collected from the index cases prior to development of severe malaria (Fig 7.10a) and matched controls (Fig 7.10b). Similarly, no correlation between both assays was also observed in the adult serum samples (Fig 7.10c).

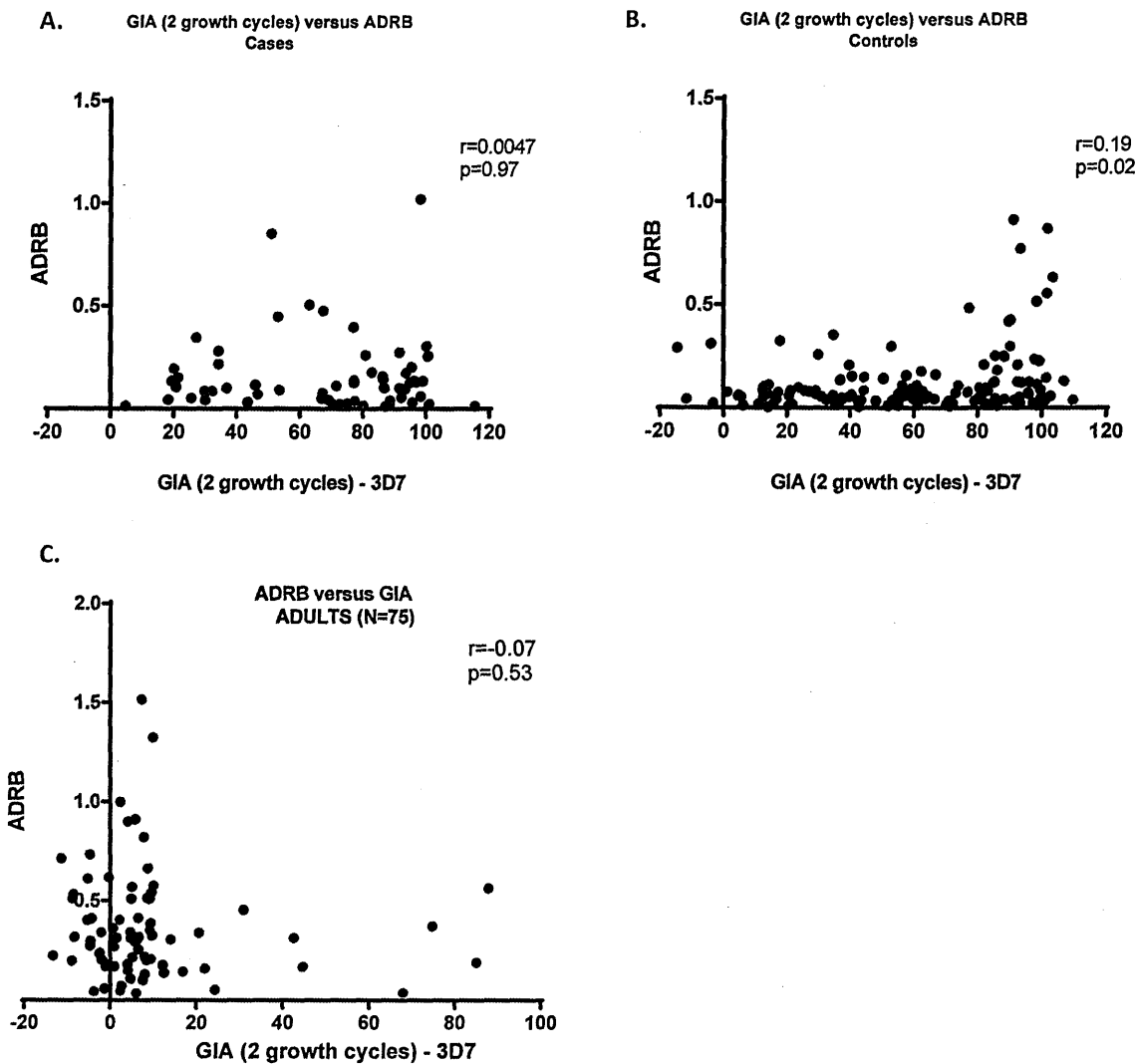


Figure 7.10 The correlation between ADRB activity and the 2-cycle growth inhibition assays in children who subsequently developed severe malaria (A) versus their matched controls (B). In comparison the correlation between ADRB and GIA was assessed in a subset of adults resident in Kilifi County (C).

7.3.11 A comparison between the breadth of antibody responses with ADRB and GIA in children aged 0-2 years

Next, the levels of ADRB and GIA were examined relative to the number of antigens that an individual made responses to (breadth of antibody responses). Only responses to one allelic type were examined for each antigen. A total of 8 children made responses to more than three antigens and these were classified as responders to 4&5 antigens. There was no clear trend between GIA levels and breadth of antibody responses (Kruskal Wallis test, $P=0.14$) (Figure 7.11a). In contrast the ADRB levels increased with increase in antibody breadth and the trend was statistically significant (Kruskal Wallis test, $P<0.001$) (Figure 7.11b).

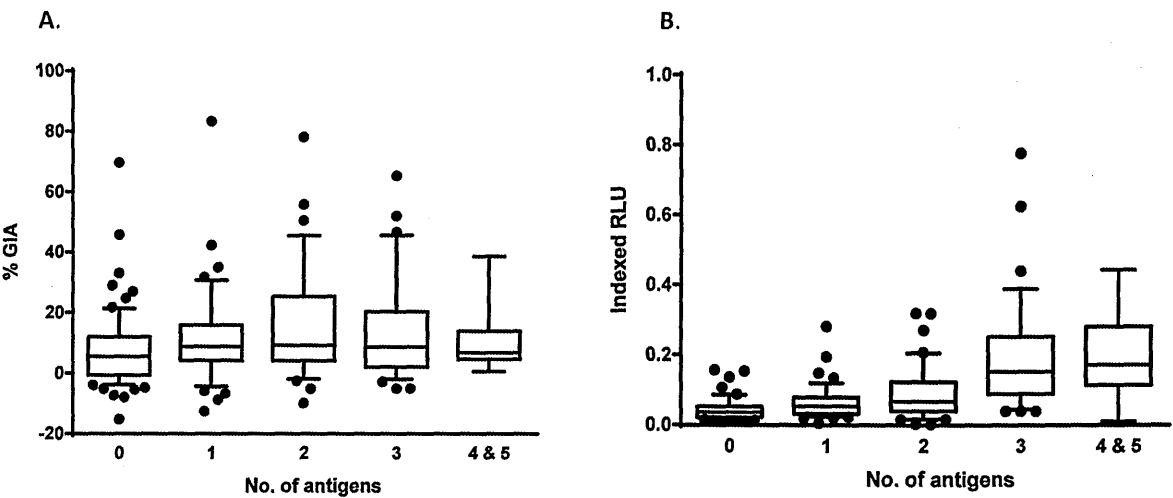


Figure 7.11. Association between breadth of antibody responses and GIA levels (A) or ADRB levels (B) in children aged 0-2 years.

7.3.12 The association between ADRB and GIA and protection against severe malaria during the first two years of life

The association between pre-existing antibodies that induce ADRB and/or mediate GIA and protection against severe malaria during the first two years of life was assessed using a conditional logistic regression model. Assays were performed using plasma collected prior to the disease episode from cases (N=42) and their corresponding controls matched on age, residence and visit date (N=127). Children were classified as responders in the ADRB assay if they achieved levels above the mean plus three standard deviation values of thirteen UK adult sera. Positive GIA responders were classified as individuals who had GIA levels above the median level. Children classified as responders in the ADRB and GIA assays alone had a reduced odds of developing severe malaria (OR 0.51 95%CI (0.14 – 1.88), p=0.319 and OR 0.55 95% CI (0.26 – 1.20), p=0.136), although the estimates were not statistically significant.

A more important and striking observation was that children who had antibodies that mediated both mechanisms had a significant reduction in odds of developing severe malaria during the first two years of life (OR 0.07; 95% CI 0.006 – 0.82; p=0.035).

7.4 Discussion

The rapid development of blood-stage vaccine candidates has been hampered in part by the lack of suitable pre-clinical screening methods and the prohibitive cost of conducting vaccine trials. The evaluation of *in vitro* functional assays that correlate with *in vivo* protection (both naturally-acquired and vaccine-induced) could accelerate the down-selection of potential candidate antigens to be put forward for testing in the clinic. The most advanced candidate antigens that have been tested in malaria endemic areas were partly selected based on the ability of antibodies induced against these antigens to neutralize parasite growth in the widely used GIA assay. For example, AMA1, a leading candidate antigen was shown to induce potent GIA in *Aotus* monkeys (Dutta et al. 2009) and in Phase 1 vaccine trials in malaria naïve adults (Polhemus et al. 2007). However, the vaccine only conferred strain-specific efficacy when tested in children living in a malaria endemic area (Thera et al. 2011) raising the question whether this assay serves as an accurate correlate of protection in individuals continuously exposed to multiple parasite strains.

Similarly several immuno-epidemiological studies correlating GIA with protection have yielded conflicting results (reviewed in (Duncan et al. 2012)), although there is considerable heterogeneity in the GIA methodologies utilized, transmission intensities in different settings, age groups tested and outcome measures examined. Here, two GIA assay methods were assessed to confirm whether, in my study, the GIA assay methodology and number of growth cycles, (factors which potentially influence the outcome of the assay) affected the inhibitory activity of sera collected from infants aged 0-2 years and adults resident in Kilifi County. I show that GIA activity in both children and adult sera is significantly enhanced in a two-cycle assay compared to a single-cycle assay as previously reported (Dent et al. 2008), possibly due to the amplification of the inhibitory effect of antibodies in the second cycle. My findings of higher median levels in the two-growth cycle assay compared to the one-growth cycle assay

are in contrast to those reported by Dent *et al*, where they showed no difference between the median GIA levels in the two assays (Dent et al. 2008). In their study they suggest that this could be explained by the wider range of responses seen in the pLDH assay compared to the FACS assays but I found no evidence of this in my setting as the range of responses by both assays was similar. I provide evidence that differences in assay methodology significantly impact on the levels of GIA reported in different settings, and there is need for standardization across laboratories to enable accurate comparison of findings. For instance, GIA assays adopted by different laboratories should be validated against the well-established GIA methodology outlined by the GIA assay reference center at the US National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health.

GIA levels were significantly higher in adults compared to infants in the two-growth cycle assay. These findings suggest that GIA levels in very young infants (0-2 years) are generally low and this is independent of the number of growth cycles. In contrast, lower GIA levels were observed in adults compared to children in the one growth cycle assay, consistent with a previous study (Dent et al. 2008) that used a similar methodology but tested slightly older children (1-4 years). The range of GIA observed in the two-growth cycle matches what has been reported in other studies more closely, for both adults (Dent et al. 2008, Crompton et al. 2010b) and children aged less than 12 months (Wilson et al. 2013). Studies that have compared GIA levels in adults versus children have examined a wider age classification of children ranging between 1-4 years (Dent et al. 2008), 2-5 years (McCallum et al. 2008), 1-8 years (John et al. 2004) and 8-14 years (Courtin et al. 2009) and reported relatively higher median GIA levels in children. It is difficult to compare my findings with these studies since the children examined here are aged less than 2 years.

In both children and adults, there was a negative correlation between GIA activity and antibody levels against all antigens tested as previously reported in other studies (John et al.

2004, McCallum et al. 2008, Rono et al. 2012, Dent et al. 2008). The obvious explanation for this is that the antigens studied here are not targets of naturally acquired neutralizing antibodies. This proposition has been challenged by findings from vaccine trials and animal model studies showing a clear correlation between antibody titres and GIA. It is likely that adults and children in endemic areas acquire blocking antibodies that interfere with the function of neutralizing antibodies (Miura et al. 2008b, Miura et al. 2011) and this could partially explain the lack of a positive association between antibody titres, age and GIA. My findings support the notion that GIA alone may not be a useful tool for screening targets of naturally acquired immunity. An important finding here and in one other study (Wilson et al. 2013) is that although studies report acquisition of growth inhibitory antibodies at an early age, this certainly excludes children aged 0-2 years. The low median GIA levels seen in children studied in this cohort could be explained by inadequate exposure and low host age. To the best of my knowledge, this is the first demonstration of the relationship between GIA and protection against severe malaria in children and we found no evidence that this mechanism was associated with a significant reduction in odds of developing severe malaria in analyses of both the one and two growth cycle GIA assays. This result is not surprising as the median GIA level in this cohort of children was very low.

The ADRB assay that has recently been described as a potential correlate of naturally acquired immunity (Joos et al. 2010) was also assessed in this chapter. I show that ADRB activity was mediated specifically by PEMS opsonised with serum from malaria exposed donors and the activity using coated PEMS as opposed to PEMS in solution, was more reproducible and eliminated non-specific reactivity. I also demonstrate that although there is donor-dependent variability in ADRB activity from different neutrophil donors in terms of the absolute RLU values, the ADRB activity is comparable when indexed relative to a positive control serum sample hence eliminating the variability and justifying the use of different independent donors

across experiments. The assay requires a minimal amount of serum, which supports its uptake for high-throughput screening in cohorts of individuals living in malaria endemic areas.

However, a few limitations were encountered during assay development. First, I observed substantial variability of different PEMS preparations on ADRB activity, which was minimized by obtaining a large yield of PEMS from one large volume of culture. This was sufficient to screen one hundred Kilifi adult samples and two hundred infant sera in duplicate. This limitation could be overcome by adopting the recently described merozoite isolation, purification and quantification method (Boyle et al. 2010), which yields pure merozoites and possibly eliminates majority of the red blood cell debris. My attempt to adapt and optimize this technique yielded insufficient merozoite yields and further optimization of this isolation method is required. Second, a substantial degree of interassay variability was noted when a similar set of serum samples were assayed on different days. Despite this, I would argue that the distinction between responders and non-responders (based on a cutoff of mean plus three standard deviations of malaria naïve sera) was consistent across the different experiments. Comprehensive validation of the assay to overcome this variability and enhance reproducibility is ongoing (Llewellyn *et al.*, manuscript in preparation).

With the exception of MSP-1₁₉ and *PfRh2*, ADRB activity and IgG levels to AMA1, MSP-2, MSP-3 and GLURP were positively correlated in both children and adults but the correlations were moderate suggesting that these antigens are potential targets of antibodies that mediate ADRB activity but do not on their own entirely contribute to this mechanism. The finding that ADRB activity levels increased with increasing breadth of antibody responses supports the hypothesis that multiple, as opposed to single, merozoite targets are more important for this response. Identifying the antigen combinations associated with ADRB activity will improve our understanding of acquisition of naturally acquired protection. Based on the set of conditions optimized for the ADRB assay, ADRB activity in sera collected prior to the disease

episode from children who subsequently developed severe malaria and their corresponding controls was similar and I found no evidence of a significant association of this mechanism with a reduced odds of developing severe malaria although the trend was towards reduced risk. Cytophilic antibodies, which bind Fc-receptors with a much greater affinity, have been shown to mediate ADRB activity in both humans (Joos et al. 2010) and mice (Llewellyn et al. 2014). However, I did not test the relative contribution of antibody subclasses in mediating ADRB in this chapter.

Overall, the most striking finding was that children who had both ADRB and GIA activity had a significant reduction in odds of subsequently developing severe malaria during the first two years of life. These findings suggest that antibodies are likely to protect young children from severe episodes of malaria via multiple mechanisms and the assessment of the breadth of functional immune responses may be more valuable for evaluating protective immunity against severe malaria than single mechanisms or serological measures of antibody titres. To the best of my knowledge, this is the first demonstration that these two functional antibody assays are prospectively associated with protection from severe malaria. Further studies on these responses in different populations are therefore needed to validate these findings.

In addition, the wider application of these assays in other clinical outcomes such as uncomplicated malaria episodes and parasitization would also be useful. My observation of a lack of correlation between the ADRB and GIA assay outputs, which measure distinct antibody-mediated anti-parasite effector mechanisms, together with the demonstration of an increase in ADRB activity with increasing antibody breadth, highlights the involvement of separate and possibly multiple protective antigenic targets of both assays. Screening these targets will inform vaccine development efforts and improve our understanding of targets of NAI.

In summary, my findings provide useful insights into potential mechanisms that protect against severe malaria in young infants, and encourage the assessment of various functional assays that measure distinct components of the antibody responses.

CHAPTER EIGHT

Conclusions and recommendations

8.0 Summary of findings

Antibodies contribute a large part of the natural defence against malaria episodes as demonstrated by passive transfer experiments, but a major issue in understanding naturally-acquired immunity to malaria, as well as the development of vaccines, has been the lack of agreement on the protective effects of different antibody responses to malarial antigens and the lack of focus on responses associated with immunity against severe malaria. The analyses and experiments presented in this thesis aimed to address the above issues and I summarize the findings for each of them.

First, the finding in our own setting that antibodies to merozoite targets (that had previously been associated with protection in a cohort of children recruited during a high transmission period) were not associated with protection in a separate cohort (recruited several years later during a period of moderate malaria transmission) was intriguing, and provided the impetus for resolving this paradox. I used a common reference standard reagent to quantify and standardize measurement antibodies to a panel of merozoite antigens in both cohorts and I have demonstrated in chapter four that: -

- Age-matched children recruited in the moderate transmission cohort had significantly lower antibody titres to the antigens tested compared to those recruited in the high transmission cohort and this was a possible explanation for the lack of protection seen in the moderate transmission cohort. These findings drew my attention to the importance of generating not only a detectable antibody response, but a high titre response. Previously, descriptions of so-called “high titre responses” have largely been

study site specific, but here I have provided estimates of the specific levels that should be achieved and evaluated in other settings.

- Achieving high antibody titres above specific threshold concentrations was associated with a reduced risk of developing clinical episodes of malaria. I have provided evidence for this by showing that despite the relatively lower antibody titres in children recruited in the low transmission cohort, those who achieved antibody levels above the specific threshold concentrations defined in the high transmission cohort had a reduced risk of developing a clinical episode of malaria.
- Intriguingly, threshold concentrations derived independently in three independent cohorts were comparable lending support to a possible clinical and/or biological relevance. The proposed threshold levels could be useful correlates of protection against clinical episodes of malaria. Further, the anti-parasite effector mechanism(s) conferred in the individual by achieving these threshold titres remains to be determined, but should help to further support the biological plausibility of these thresholds.

Second, there is a paucity of data on protective mechanisms and targets of immunity to severe malaria. Whilst this, in part, results from the logistical challenges of following up a fairly large number of children for a relatively rare outcome, the few published studies of the association between antibody responses to blood-stage antigens and protection from severe malaria have yielded inconclusive results. Using a longitudinally monitored birth cohort of children aged 0-2 years, the findings presented in this thesis demonstrate that: -

- Anti-merozoite antibodies are infrequently found in children below the age of two years with the exception of those aged less than 6 months.
- Children with asymptomatic infections were relatively few and such infections were characterized by transient peaks in antibody levels. Importantly, there was no

difference in antibody titres at all time-points, between cases and age-matched controls to all the antigens tested.

- There is a lack of association between antibody responses to a panel of merozoite antigens and development of severe malaria during the first two years of life with the exception of IgG to AMA1 alleles. Anti-merozoite antibody levels may thus have been too low to confer any significant protective effect.
- There is a consistency of the association between antibodies measured at a single time-point versus multiple-time points prior to a disease episode, hence supporting the utility of single time-point antibody measurements in immuno-epidemiological studies assessing the relationship between antibodies and protection against disease in this age group of children.
- The first systematic analysis of the longevity of maternal antibodies against a panel of merozoite antigens. The half-life estimates are informative in deciding when to administer malaria vaccines to this group of children, based on the evidence that maternal antibodies may interfere with a child's ability to mount an effective response vaccines (reviewed in (Siegrist 2001) and (Siegrist 2003)).
- The rate of decay of maternal antibodies is dependent on the concentration of antibodies present at birth. Children who acquired high titre antibodies from their mothers had the highest decay rate. Such kinetics of antibody decay suggest that malaria vaccines that protect via induction of high titre antibodies will require constant boosting in this group of children in order to maintain their protective levels.
- Reduced susceptibility to severe malaria was associated with a combination of antibodies that can limit growth of parasites as measured by both GIA and ADRB assays. These assays will be useful as potential correlates of protection against severe malaria. These findings also provide evidence that multiple, as opposed to single, functional mechanisms may be more important in protection from malaria.

Our previous work demonstrating that children who developed severe malaria had more asymptomatic infections and harbored diverse parasites compared to their age-matched controls (Lundblom et al. 2013), reflects a higher degree of exposure in this group of children at an age when anti-merozoite antibodies have not attained concentrations required for protection against severe malaria. I have extended these findings by showing that age-matched controls were more likely to have a combination of antibodies that mediated GIA and ADRB compared to cases, lending support to a role of antibody-mediated function being more important for protection against severe malaria as opposed to the ability to generate high titre responses to these antigens. The question of what might be the targets of these functional antibodies is of considerable importance and interest towards identifying potential vaccine targets. More detailed studies are needed to elucidate the merozoite targets of functional antibodies.

The studies presented in this thesis thus enhance our knowledge of the acquisition of immunity to malaria and provide insights that may be valuable for advancing malaria vaccine development and testing.

8.1 Recommendations for future studies

This work could be extended in future studies by: -

- i) Examining the threshold concentrations proposed in this thesis in other settings with varying transmission intensities and converting these into absolute concentrations for direct comparison in vaccine trials.
- ii) Measuring responses to additional and novel merozoite antigens, or antigens expressed by other parasite stages. Given the complexity of the malaria parasite, it is likely that a broad repertoire of immune responses targeted against multiple antigens are required to achieve immunity to malaria. Recent advances in genomics and protein expression

systems have facilitated the identification and expression of a broader panel of novel antigens or combinations of antigens that are targets of protective cellular (Cardoso et al. 2011) and antibody (Raj et al. 2014, Richards et al. 2013, Crompton et al. 2010a, Doolan et al. 2008, Osier et al. 2014b) responses. The work presented in this thesis could be extended further by probing whole proteome arrays with pooled plasma from cases and corresponding controls who do not develop severe malaria. Such a screening approach would accelerate the identification of combinations of antigens that are targets of protective antibody responses in my setting.

- iii) Adoption of unbiased approaches such as systems biology to screen immunological parameters associated with resistance or susceptible to disease. The systems approach combines predictive computational modeling with high-throughput cellular and molecular assays such as whole-blood transcriptomics, multiple-plasma cytokine/chemokine assays, multicolor flow cytometry and antibody profiling using proteome-based chips. A few studies have adopted this approach and identified early molecular signatures that predict/correlate with immune responses induced after vaccination against yellow fever (Querec et al. 2009, Gaucher et al. 2008), influenza (Nakaya et al. 2011) and meningitis (Li et al. 2014).

Thus far, only one study has utilized a systems biology approach to analyse immune responses induced following natural exposure to malaria infection in a longitudinally monitored cohort (Portugal et al. 2014). Adoption of a similar approach in my setting and others could provide a more comprehensive definition of immune responses that are protective from those that are markers of exposure and hence improve our understanding of the mechanisms associated with NAI.

- iv) Distinguishing responses and targets associated with protection against the individual symptoms of severe malaria. Due to the relatively small numbers of cases with single

symptoms of cerebral malaria, severe malaria anemia and respiratory distress, I did not assess them in this thesis as separate outcomes of severe malaria.

- v) Evaluating the combination of GIA and ADRB assays in protection against the outcomes of severe malaria and uncomplicated malaria in other settings. In addition, evaluate the merozoite targets of these mechanisms.

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